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Isotopic biomarker discovery and application in translational medicine

Henry K. Bayele[1], Arturo Chiti[2], Rodney Colina[3], Octavio Fernandes[4], Baldip Khan[5], Rajagopal Krishnamoorthy[6], Hilal Özdağ[7] and Rose Ann Padua[8]

[1] Department of Structural & Molecular Biology, University College London, Gower Street, London WC1E 6BT, United Kingdom
[2] U.O. di Medicina Nucleare, Istituto Clinico Humanitas, via Manzoni, 56, 20089 Rozzano (MI), Italy
[3] Centro de Investigaciones Nucleares, Facultad de Ciencias, Universidad de la República, Mataojo 2055 Esq. Igua 4224, Montevideo 11400, Uruguay
[4] Laboratory of Molecular Epidemiology of Infectious Diseases, Departamento de Medicina Tropical, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Avenida Brasil, 4365 Caixa Postal 926, 21045-900 Rio de Janeiro, Brazil
[5] Nuclear Medicine Section, Division of Human Health, International Atomic Energy Agency, PO Box 100, 5 Wagramerstrasse, Vienna A-1400, Austria
[6] INSERM, U 763, Hôpital Robert Debre 48, Boulevard Serurier, 75019 Paris, France
[7] Institute of Biotechnology, University of Ankara, Beşevler 06100, Ankara, Turkey
[8] INSERM UMRS 940, Université Paris 7 Denis Diderot, Faculté de Médicine, Institut Universitaire d'Hématologie, AP-HP, Hôpital Saint-Louis, 1 Ave Claude Vellefaux, 75010 Paris, France

Rational drug discovery relies on pathognomonic molecular reporters of disease or biomarkers. Therefore biomarkers contain relational or contextual information about disease pathophysiology. Two broad pathways can be taken to identify biomarkers: a 'top-down', holistic approach that makes no assumptions about biomarker type, or the 'bottom-up' approach, which is hypothesis driven and relies on *a priori* information. Both approaches involve parallel or sequential methods that include genomic and proteomic profiling. Biomarker discovery and translational medicine owe much to isotopic techniques because these provide near-real-time information about disease status as diagnostics, in drug delivery and for monitoring treatment. Here, we provide an overview of recent developments and some insight into the future role of isotopes in biomarker discovery and disease therapy.

Introduction

To rationally design new drugs, molecular medicine requires 'readouts' or 'reporters' that indicate not only normal biological processes but also incipient disease or the risk of disease and its progression [1]. Biomarkers fulfill this role at many varied and context-dependent levels. By definition, they are dynamic and informational molecules whose identities range from genes to metabolites. For biomarkers to have any diagnostic value, they would have been validated through a series of stringent molecular methods so that any time they are identified, they would be pathognomonic of a particular biological state or disease. In other words, they must be reliable at every level, including lab-to-lab reproducibility. They will report not only on disease presence but also on its response to treatment. Biomarkers, therefore, are important surrogates for whole-body monitoring in molecular medicine. Their predictive value largely drives their current demand in translational, preventative and personalized medicine, particularly because they have the potential to eliminate trial and error in clinical practice and drug development. Because of inter-individual pharmacogenetic variation in drug response, the attrition rate of clinical drug trials is ~90% [2]; therefore, the search for veritable biomarkers for prototypical human diseases such as cancer, inflammation and age-related diseases (including neurodegeneration) has never been more urgent. However, the path to biomarker identification can be long, arduous and expensive, with no guarantees that they can be sufficiently validated as markers of disease. Nonetheless, those biomarkers that pass stringent validatory tests are a boon for the pharmaceutical industry, especially for high-throughput assays in drug discovery and as diagnostics.

Corresponding author:. Bayele, H.K. (bayele@biochem.ucl.ac.uk)

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Some of the crucial paths to biomarker discovery and validation require isotopic methods because of their superior sensitivity over non-isotopic techniques. Here, we assess some of these procedures in both qualitative and quantitative biomarker discovery and the potential application of these biomarkers in drug discovery and translational medicine.

Quantitative 'omics'

Biomarker discovery can include a holistic or top-down approach that involves whole-organismal biology (genomes or proteomes) or specific disease pathways, such as cancer and inflammatory disease. This method is essentially a 'black box' approach that makes no assumptions about what the biomarkers are. By scanning a whole organism or pathway using a few criteria (e.g. what gene expression profiles are changed in disease), such biomarkers can be sampled from the gene to the protein level and subsequently tracked by molecular imaging methods (Figure 1). Although a great deal of potential biomarker information might accrue from this approach, some of that information might be confounding or spurious. The bottom-up approach, by contrast, is largely hypothesis driven and relies on *a priori* knowledge of what or where the biomarkers might be; this might be pathogen virulence factors or nodal proteins from which several pathways might ramify. The bottom-up approach is target orientated and is not subject to the complexity of the top-down approach. Similar to the top-down approach, however, potential biomarkers can be tracked from the cognate gene to protein levels and beyond (e.g. in diagnostics and therapeutics) by isotopic methods. There might also be some overlap between top-down and bottom-up approaches, such as in a particular disease pathway or cell type. Biomarkers that are identified by either route might be of genomic or proteomic origin, although this definition is broad and a gross oversimplification; this is because new biomarker types and methodologies for their detection are constantly evolving, as is discussed below.

Genomic biomarkers

The ultimate aim in functional genomics is to deconstruct or tease apart gene interaction networks to understand physiological or disease pathways. The complete sequencing and annotation of the human genome makes this possible and provides a trove of genomic biomarkers that might be specific to certain diseases. Functional genomics also ushers in a new era of personalized treatment for complex diseases such as cancer, diabetes, autoimmune diseases and age-related diseases. Each of the 20,000–25,000 protein-coding genes in the human genome [3] and their derivatives (e.g. mRNA, splice variants, polymorphisms and mutations) are

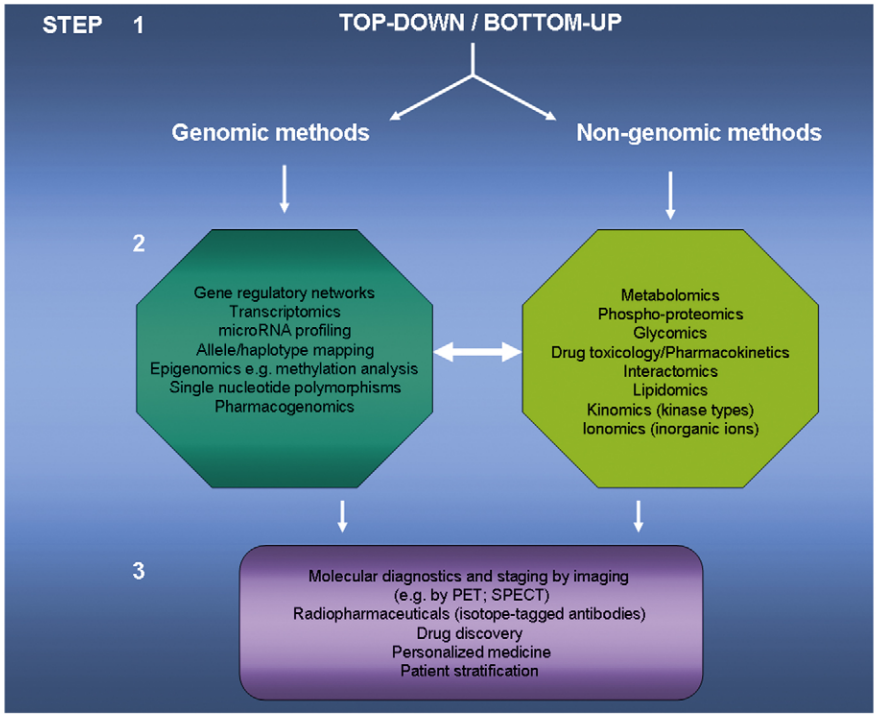

FIGURE 1

Pathways for biomarker discovery and application. As a first step (step 1) in biomarking, one of the two main pathways might be taken: a holistic, top-down approach, or a bottom-up approach. Whereas the former might involve whole-organismal biology (genomes or proteomes) and specific disease pathways and is not predicated on any assumptions, the bottom-up approach is hypothesis driven based on *a priori* information. In step 2, specific approaches are applied to answer the primary question in step 1. For genomic biomarking, this might involve ancillary procedures such as isotopic hybridizations, S1 nuclease mapping, DNA footprinting, mutation analysis and microarrays. Principle proteomic or non-genomic procedures (which are described in detail in the text) might also be aided by ancillary methods, such as isotopic cell-free *in vitro* protein expression and metabolic labeling (e.g. with ^{15}N, ^{13}C and ^{35}S), 2D polyacrylamide gel electrophoresis, liquid chromatography and mass spectrometry. Both genomic and non-genomic approaches employ bioinformatics for biomarker identification. The final step (step 3) involves translational application of the identified biomarkers in therapeutic drug discovery and diagnostic imaging or simply for patient stratification.

potential biomarkers for such diseases. For example, specific mutations in the epidermal growth factor receptor (EGFR) gene, *p53* and *K-ras* have been linked to various types of cancer [4–6]. Thus, genomic biomarkers include genes (and their allelic polymorphisms), expressed sequence tags, microRNA and mRNA (and their splice variants), single-nucleotide or copy number polymorphisms, epigenetic modification status, and haplotypes. However, not all of these have diagnostic significance, thus requiring their stratification into which ones are veritable and which are simply due to noise (e.g. spurious changes in gene expression as a result of transient changes in the genomic environment). Hitherto, genomic biomarkers have been identified by hybridization with, for example, ^{32}P-labeled DNA or RNA probes. This has been superseded by microarrays [7], which have immensely simplified biomarker identification through multiplexed genome-wide gene expression analysis. Microarrays, therefore, have been used extensively to search for diagnostic and/or prognostic markers for disease staging, monitoring patient responses to treatment, and finding novel therapeutic targets.

There are limits, however, to which genomic biomarkers can be applied, primarily because gene–environment interactions, epistasis and even gene–nutrient interactions can grossly skew the representation or expression profile of certain genes in different individuals. The stochastic nature of gene expression also means that expression profiles might not necessarily be reflective of the full complement of 'expressible' proteins in either space or time. Furthermore, transcriptome analysis is not fully predictive of the proteome of a specific cell or tissue, owing to the presence of non-coding RNAs, alternative splicing and differential rates of mRNA translation or degradation. For example, approximately 70% of human genes have alternative splice forms, resulting in differential gene expression and the production of structurally and functionally distinct proteins from a single gene (for a review, see Ref. [8]). Chen *et al*. [9] have also demonstrated heterogeneity in a large number of differentially expressed proteins in gastric cancer cells, showing that mRNA profiles do not always correlate with protein profiles within a cell. For these reasons, protein biomarkers are preferable because they are more reliable indicators of temporal cell status at different levels in both health and disease.

Proteomic biomarkers

Proteomic biomarker types

Proteomics is an exploratory tool to determine the full complement of expressed proteins within cells and/or tissues (or even whole organisms) at any point in time. This then provides a basis for understanding how these proteins might interact functionally and combinatorially in defined pathways. The types of proteins that might be profiled include – but are not limited to – cell-surface proteins, antibodies and antigens [1]. They might also be defined by their post-translational modification status (e.g. phosphorylation or glycosylation). The phosphorylated form of a protein might indicate activation; this activation state might itself be a biomarker. Similarly, glycosylated EGFR isoforms and other glycans (including serum glycoproteins) have been identified as markers for prostate, breast, pancreatic and lung cancer [10–13]. Invariably, these proteins or their derivatives are identified by isotopic incorporation during *in vitro* culture and further characterized by mass spectrometry (MS). By combining bioinformatic algorithms and sequence database analysis, putative biomarkers can be identified with a degree of confidence [14–16].

General methods in proteomic biomarker discovery

Quantitative proteomic profiling determines the differences in protein expression between samples under various treatment regimes or biological states (e.g. normal or diseased tissue). To enable this, several isotopic MS methods have been developed (Figure 2). These include the stable isotope labeling with amino acids in cell culture (SILAC) protocol. This involves metabolic incorporation of 'light' or 'heavy' forms of amino acids into nascent proteins and stable isotopes such as ^{2}H, ^{13}C and ^{15}N and their detection by liquid chromatography (LC)–tandem mass spectroscopy (MS/MS) after proteolytic cleavage of the peptides [17]. This protocol has now also been adapted for *in vivo* use in the ^{15}N-labeled rat and the SILAC mouse. In the latter, mice are fed on diets enriched with $^{13}C_6$-lysine or $^{12}C_6$-lysine over several generations and isotopic incorporation into proteins is determined by MS [18,19]. Although it might have its own limitations (e.g. differences in the rates of metabolism of the different isotope diets), this approach should enable the elucidation of proteomic differences in health and disease or between normal metabolic and disease pathways.

Currently, one of the major concerns in proteomics is the comparative analysis of 2D gel images. This is fraught with difficulties because there are variations between spot intensities, even for identical spots on parallel gel runs; protein patterns are never perfectly superimposable; and protein detection can often be obscured by high-abundance proteins such as albumin and immunoglobulins. The protein standard absolute quantification developed by Brun *et al*. [20] includes synthetic proteins labeled with [$^{13}C_6$, $^{15}N_2$] L-lysine and [$^{13}C_6$, $^{15}N_4$] L-arginine as internal standards to accurately quantify proteins in 2D gels, and the co-electrophoresis of fluorophore-labeled samples using the 2D fluorescence difference gel analysis technology [21] can circumvent some of these problems. The differential gel exposure method uses radioisotopes in place of fluorescent dyes. Here, samples are differentiated from each other by the incorporation of two different isotopes (e.g. ^{14}C and ^{3}H) *in vivo* [22]. This approach was used to identify differentially expressed proteins as biomarkers in renal cell carcinoma using ^{125}I and ^{131}I followed by 2D analysis and differential radioactive imaging [23]. Immunodepletion of albumin and IgGs can improve the detection of serum or plasma proteins that are otherwise undetectable [24].

One of the attractions of LC–MS/MS is the ease and sensitivity of biomarker separation because it can resolve and identify biomarkers in exceedingly low femtomolar concentrations [25–28]. The isotope-coded affinity-tag (ICAT) peptide labeling method can measure such quantitative differences between the levels of protein expression [29]. By differentially tagging cysteines in proteins with stable heavy and light isotopes of two different cell systems, biomarkers can be identified from highly complex mixtures of peptides by LC–MS/MS based on the differences between the isotopically 'light' or 'heavy' forms. The ratio of intensities of the peptide peaks in a given mass spectrum gives a relative ratio of abundance of the two species. Although the ICAT method is an ideal method for accurately quantitating low copy number biomarkers, it is not amenable to large-scale or high-throughput

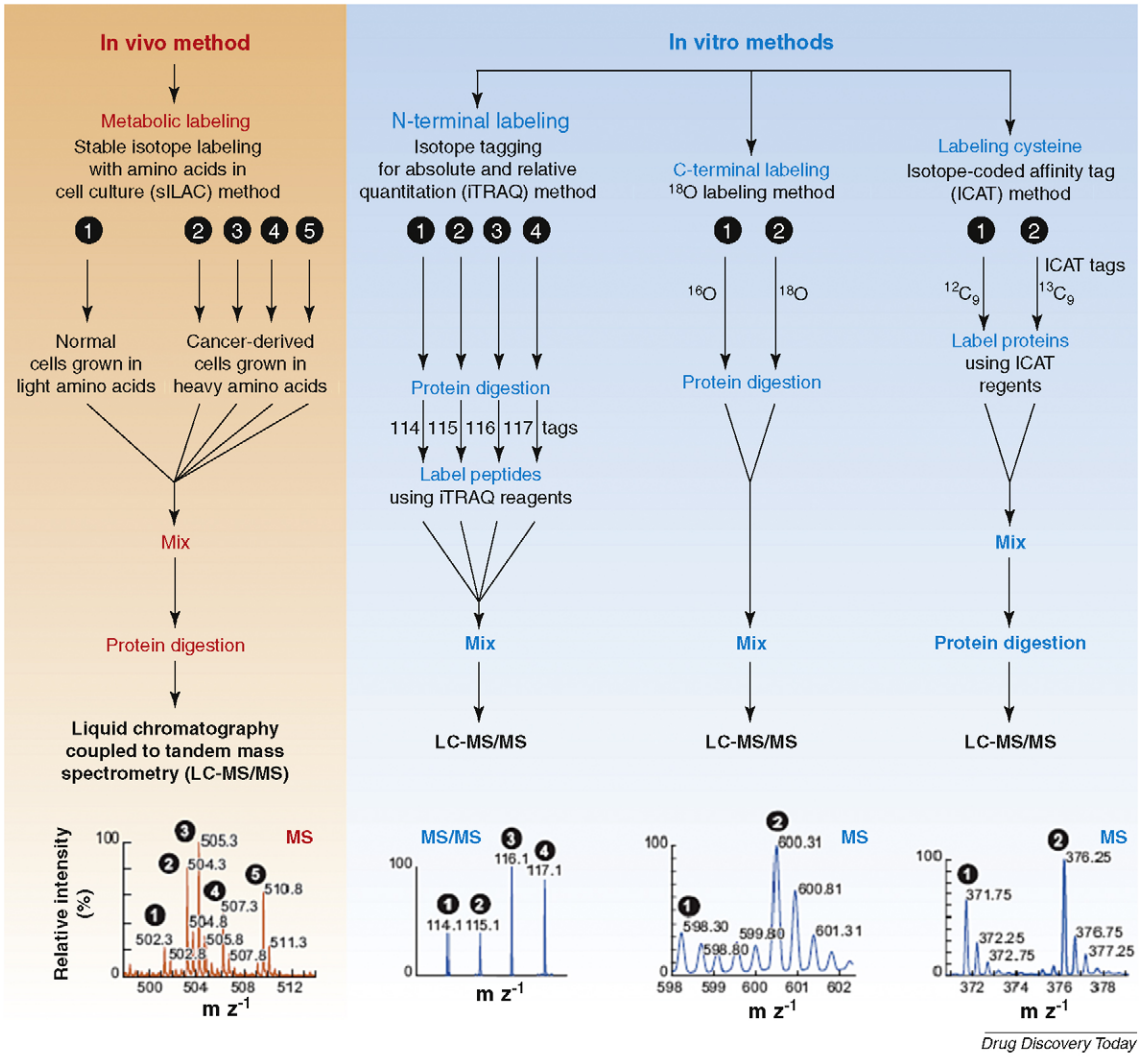

FIGURE 2

Stepwise biomarker identification by *in vivo* and *in vitro* isotopic labeling. For *in vivo* labeling, isotope-tagged amino acids are metabolically incorporated into *de novo* synthesized proteins in normal or cancer cells. Proteins might also be labeled *in vitro* using isotopically labeled amino acids in cell-free extracts. Proteins labeled by either method are identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after proteolysis. Reproduced, with permission, from Ref. [16] (http://www.annualreviews.org). Please see Ref. [35] for additional proteomic approaches. Details of the methods are described in the text under 'Proteomic biomarkers'.

quantitative analysis. Its reliance on cysteine labeling also means that target proteins or biomarkers that are devoid of this residue cannot be detected.

The isobaric tags for relative and absolute quantitation (iTRAQ) technique [30] require prior N-terminal labeling of the target proteins from various sources, cell types or treatments; these are then digested into peptides, which are subsequently labeled with isotope tags of different molecular masses. After mixing, the peptides are resolved by LC–MS/MS, and by comparing the mass spectra, it is possible to differentiate between cleaved reporter isotope tag and tagged peptides and to quantify the latter relative to the tag. Peptide identity is determined by searching the databases. At present, only four isotopically unique or distinguishable iTRAQ reagents are available, but this number is certain to increase because of the potential for multiplexing or analyzing several samples in a single run. Another *in vitro* method of protein quantitation by spectral analysis employs the absolute quantification, or AQUA, method [31]. Here, proteins are incorporated with stable isotopes (e.g. ^{18}O, ^{13}C and ^{15}N) during synthesis and used as internal standards to quantify the absolute levels of post-translationally processed proteins within a mixture after digestion and LC–MS/MS.

Enzyme-catalyzed incorporation of ^{18}O can also be used to label and identify peptides from mixtures of proteins *in vitro* for quantitative proteomics [32]. This approach exploits the observation that proteolysis invariably incorporates one atom of oxygen to the C-termini of the resulting peptides; no exchange of solvent oxygen occurs in the absence of proteolysis. In this technique, a 1:1 mixture of natural and heavy ($[^{16}O]$:$[^{18}O]$) water is added to the proteins during trypsin digestion; trypsin catalyzes the incorporation of ^{18}O at the C-termini of each peptide, which are then detected by LC–MS/MS based on mass shift. This enables the

differentiation of the C-termini of peptides digested in natural water compared with isotopically labeled peptides. When proteins are digested with trypsin, Lys-C and Glu-C (protease V8) in ^{18}O, the molecular masses for the resulting peptides shift proportionately by 2 Da or 4 Da; other proteases, such as chymotrypsin, yield only 2 Da shifts because they can catalyze the incorporation of only one atom of ^{18}O per peptide. This enyzmatic labeling technique is also able to identify post-translationally modified or disulphide-bonded peptides. Using nanoflow reversed-phase LC together with MS/MS, trypsin-mediated ^{18}O labeling was used for proteomic analysis of formalin-fixed paraffin-embedded prostate cancer tissue, enabling retrospective biomarker discovery [33]. In addition, Stockwin *et al*. [34] used $^{16}O/^{18}O$ labeling to identify hypoxia-inducible proteins in malignant melanoma cells. Because hypoxia is a hallmark of many cancers, these proteins might provide new tools for cancer drug discovery and treatment.

Limitations in proteomic biomarker discovery

A major drawback in proteomic profiling and biomarker discovery is the low-level expression of target proteins and subsequent difficulties in their detection. Furthermore, proteins or peptides are not readily ionizable, making their accurate detection or measurement of their levels by MS impossible. To overcome these difficulties, internal standards labeled with stable isotopes such as ^{13}C and ^{15}N are included during analysis [25,31]. This enables extremely reliable and sensitive detection and quantitation of small differences in biomarker expression in plasma or serum in health and disease. For these techniques to fulfill their full potential applicability, however, a broad range of internal standards will be required. The methods are also limited because they require *a priori* knowledge of the protein(s) of interest to generate internal standards.

The single most important and intractable bottleneck in proteomic biomarker identification is that unlike genomic biomarker profiling, non-genomic or proteomic methods are not amenable to high-throughput analysis, often requiring extensive lengths of time to compare just two different samples or proteomes. To date, only the iTRAQ method [30] can analyze several samples simultaneously. Another confounding factor is that all of the proteomic methods involve limited proteolytic digestion of the labeled proteins. This inevitably increases sample complexity. However, the iTRAQ technology seems unhindered by this because it seems to be able to resolve discrete peptides because the isobaric masses of the reagents avoid mass spectral overlap. Despite these shortfalls, isotopic proteomic methods are adjunctive tools that would speed up biomarker discovery by enabling increased detection sensitivity and measurement precision [35].

Biomarkers of inflammatory diseases

Chronic inflammatory diseases cause a great deal of morbidity and/or mortality worldwide and include cardiovascular disease, autoimmune diseases (e.g. rheumatoid arthritis, systemic lupus erythematosus and type I diabetes), inflammatory bowel diseases (i.e. Crohn's disease and ulcerative colitis), cancer and neurodegenerative diseases. In general, these diseases result from a failure of negative control feedback mechanisms to mollify inflammatory responses to infection or injury. The delayed shutdown of these responses culminates in a sustained release of proinflammatory cytokines [36] – such as tumor necrosis factor-α, interferon γ and interleukin (IL)-6 – and chemokines, such as RANTES, IL-8 and MIP-1α, concomitant to macrophage activation at sites of injury or infection. Activated macrophages also release surface antigens (e.g. the major histocompatibility complex), reactive oxygen species, and antimicrobial peptides and proteases. These chemokines, cytokines and macrophage activation signals, therefore, become biomarkers of inflammatory disease. Although the path or initiating stimulus might differ for each disease, there is a general consensus that most inflammation markers such as IL-6 are non-specific. However, a discrete set of biomarkers that are pathognomonic of particular inflammatory disorders can be elicited. For example, high cholesterol coupled with raised C-reactive protein levels are made manifest in heart disease [37], and neurodegenerative diseases such as Alzheimer's typically have amyloid deposits or plaques as diagnostic markers [38]. Biomarkers of other conditions such as pain, neurological disorders, respiratory distress, musculoskeletal and connective tissue diseases and endocrine disorders are now being identified, but the pace of discovery here is far less rapid than in cancer and cardiovascular disease because these are more prevalent and life-threatening. Although not regarded as a disease *per se*, aging is accompanied by one or more of the above diseases; thus, by default, these molecules might also be biomarkers for aging or age-related diseases. In spite of the large number of potential biomarkers for these diseases, they can usually be identified using the same isotopic techniques (see 'Applications of isotopic biomarkers in translational medicine' section). An excellent and comprehensive compilation of biomarker resources for these diseases is also available at http://www.hks.harvard.edu/m-rcbg/hcdp/readings/Biologics.pdf.

Biomarkers of infectious diseases

Like cancer or inflammatory diseases, human pathogens also generate unique biomarkers after infection. Biomarkers of infectious diseases are important because these diseases account for a large proportion of all chronic illnesses; for example, infections alone cause 15–25% of all cancers and ~26% of all deaths worldwide [39]. Infectious disease biomarkers might include virulence genes or antibodies, which might aid disease diagnosis and staging. For example, HIV diagnosis relies on the presence of antibodies to the nef protein in patient sera, and hepatitis B or C infection can be detected by the presence of antibodies to the cognate viral antigens. Similarly, the *CagA* pathogenicity island is a biomarker for the pathogenic strain of *Helicobacter pylori*, which is associated with peptic ulcer and adenocarcinoma [40]. *H. pylori* infection might also be detected using its urease as biomarker. Detection exploits the ability of *H. pylori* urease to hydrolyze urea into ammonia and CO_2. By feeding subjects with ^{13}C-labeled urea and analyzing their breath for $^{13}CO_2$ with an infrared-^{13}C-stable isotope analyzer, infection and its transmission dynamics can be tracked [41]. For a comprehensive list of pathogen-specific biomarkers, please see the Infectious Disease Biomarker Database (http://biomarker.cdc.go.kr:8080/biomarker/biomarker_list.jsp?group=2). Although most of these biomarkers can be detected by traditional methods, such as ELISA and Western blotting, the sensitivity of these techniques can be improved by isotopic methods. These isotopes might also be used to track an infection cycle, virulence factor secretion or intracellular trafficking. This is

often achieved by metabolic pulse-labeling with ^{35}S-labeled methionine or cysteine; these isotopes are incorporated into the nascent proteins, which can then be visualized by gel electrophoresis and fluorography. This approach was used by Colina *et al*. [42] to identify interferon regulatory factor 7 as a nexus for type I interferon signaling and a marker of resistance to vesicular stomatis virus infection. Similar approaches were used to identify biomarkers of host response to viral infection (see Ref. [43] and references therein).

Applications of isotopic biomarkers in translational medicine

Isotopic biomarking methods in 'metabolomics'

Although proteomic profiling can inform us about the complement of expressed proteins within a cell, the interactions or failure thereof between those proteins along crucial metabolic pathways will ultimately determine cell fate. For example, enzymes of the glycolytic pathway or the Kreb's cycle are crucial for intermediary metabolism, producing metabolites such as ATP that can be detected by gas chromatography–MS or LC–nuclear magnetic resonance. Isotopic methods have been used for studying such metabolic processes over the years [44,45]. Thus, methods incorporating isotopically labeled substrates such as ^{13}C-glucose to study these pathways enable not only the tracking of energy flux but also the subsequent detection of glucose metabolites such as pyruvate. Innovative adaptations of these methods, including a modified ICAT, are being used for real-time monitoring of processes as diverse as gene expression dynamics *in vivo* [46], intracellular pH [47] and oxidative stress [48]. It is informative that changes in these parameters occur in cancer, age-related diseases (including neurodegeneration), the metabolic syndrome and inflammatory diseases [49–52]. In another modification of ICAT, Leichert *et al*. developed OxiCAT [48] to detect redox changes in cellular proteins during oxidative or nitrosative stress. This has important potential applications for identifying cytoprotective antioxidant molecules that could be important biomarkers of diseases that are precipitated by oxidative metabolism. The ability to monitor disease progression or treatment in real time by ICAT or its modifications, therefore, is a crucial tool.

In vivo *diagnostic imaging*

Disease therapy relies on accurate diagnosis; this is substantially aided by isotopic imaging *in vivo* [53,54]. Thus, some of the hallmarks of cancer – such as increased glucose uptake and metabolism, angiogenesis (neovascularization), hypoxia, and deregulated apoptosis – have been used as imaging tools. For example, to monitor energy metabolism in tumors, 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) is often used as a tracer [53,54] and, to date, has been the mainstay of non-invasive tumor diagnostic imaging, staging and monitoring during therapy using positron emission tomography (PET). The EGFR, which is overactive in cancerous cells, is a marker of cell proliferation and can be monitored with the radiolabeled ligands [^{18}F]MLO1, [^{11}C]MLO3, and [^{11}C]Iressa [55,56]. For hypoxia PET–computed tomography (CT) imaging, [^{18}F]fluoromisonidazole and [^{18}F]fluoroazomycin arabinoside have been employed successfully [57]. Angiogenesis can be tracked with radiolabeled RGD peptides e.g. [^{18}F]Galacto-RGD [54,58]; this binds to the $\alpha v\beta_3$ integrins which are overexpressed in cancer cells. The vascular endothelial growth factor (VEGF) family and their receptors are key regulators in tumor neovascularization. N-terminal Cys-tag-VEGF conjugates have also been synthesized to facilitate *in vivo* imaging of the tumor vasculature by single photon emission computed tomography (SPECT) or PET [59]. The ability to conjugate the Cys-tag moiety of proteins with isotopes also enables the functional *in vivo* imaging of biomarkers to disease-specific pathways and can be used to label and track any biomarker. Response to treatment can be imaged by detecting apoptosis using technetium (^{99m}Tc) conjugated to annexin V and SPECT [60] or with anti-angiogenic drugs, such as the thalidomide analog revlimid; this is monitored with tracers such as 3-[^{18}F]fluoro-3-deoxy-thymidine, ^{11}C-thymidine, ^{11}C-methionine and ^{18}F-FDG [53,61]. The combination of PET and CT [62] provides improved functional and morphological definition in real-time for patient management [63]. These methods, therefore, have been invaluable diagnostic and prognostic tools, not only in cancer but also in other prototypical human diseases, including cardiovascular [60,64] and neurodegenerative diseases [65]. For example, PET imaging of ^{18}F-fluoro dihydroxyphenylalanine and ^{11}C-raclopride uptake can be used to determine dopamine transporter function to assess neurotransmission, motor control and cognition in Parkinson's disease [66].

In an adaptation of PET to use on small animals, or microPET, Radu *et al*. [67] recently identified 1-(2′-deoxy-2′-[^{18}F]fluoroarabinofuranosyl) cytosine ([^{18}F]FAC) as a PET probe to study the purine salvage pathway in myeloid cells. Because these cells are defective in *de novo* purine and pyrimidine synthesis, it was possible to study lymphoid organ and innate immune functions by tracking myeloid cell activation. This, therefore, provides a method for monitoring innate immune responses to infection, inflammatory diseases and cancer (particularly during the active phase of the disease) and during resolution or treatment. [^{18}F]FAC-PET has been applied, for example, in monitoring systemic autoimmunity and its response to immunosuppression [67]. To enable intracellular pH measurements, Gallagher *et al*. [46] applied magnetic resonance spectroscopy coupled with dynamic nuclear polarization to show how isotopes can be used to monitor changes in pH *in vivo*. In other words, tissue or cellular pH can now also be classified as a veritable biomarker. This is important because large variations in pH usually occur in cancer, anoxia (e.g. in ischemic heart disease) and inflammatory diseases. Using hyperpolarized ^{13}C-labeled bicarbonate (an endogenous cellular buffer), they showed that pH differentials between normal tissue and tumors can be measured by molecular imaging in mice. This technique potentially couples non-invasive tracking not only to redox changes or acid–base balance *in vivo* but also to disease progression and during the course of treatment [68].

Protein–protein interactions can also be imaged *in vivo* and might be useful for delineating biological processes and how derangements in these interactions might cause disease. This might provide a basis for improved drug design and therapeutic intervention where such drugs might interfere with receptor–ligand interactions, for example. In particular, ^{13}C-arginine has been used to study the EGFR activation pathway [69]. Non-invasive imaging of such interactions *in vivo* would enable real-time monitoring of the effect of drugs or, indeed, simply determining the interaction dynamics between any select set of 'drugable'

targets (e.g. G-protein-coupled receptor–ligand interactions) because these are involved in diverse disease and biological processes ranging from hypertension [70,71] to satiety [72,73].

Gene therapy

This is an area that could benefit from isotope application. As an example of this, micro-PET was used to track time-dependent and pulsatile expression of the herpes simplex virus 1 thymidine kinase gene (*HSV-tk*) in mice [45] (Figure 3). In rat models with human tumor xenografts, micro-PET was also used for monitoring the expression of p53-dependent genes with the thymidine kinase construct *Cis-p53TKGFP* as reporter. *HSV-tk* and *Cis-p53TKGFP* expression were determined by injecting the animals with the substrate analogs 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)guanine and 2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-[^{124}I]iodouracil, respectively [45,74]. Similar methods and radiolabeled substrate analogs have been established to detect the expression of HSV-TK or its mutant HSV-sr39tk [74], the dopamine D_2 receptor gene for imaging brain tumors using 3-(2′-[^{18}F]-fluoroethyl)spiperone as tracer [75], the type 2 somatostatin receptor gene that is imaged with ^{188}Re or ^{99m}Tc-labeled somatostatin peptide P829 [76], and the human sodium-iodide symporter gene that can be imaged with ^{121}I/^{123}I/^{124}I/^{131}I and [^{99m}Tc]O_4 as reporters [74,77,78]. The ability to track gene expression *in vivo* with radioisotopes by PET has been used in humans for monitoring tumor response to gene therapy [79]. When recombinant adenovirus expressing thymidine kinase was transduced into hepatocellular carcinoma patients, thymidine kinase expression could be detected by PET using [^{18}F]9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)-guanine; transgene expression occurred only in tumors, not in juxtaposing normal tissue. This approach is crucial for monitoring tumor growth, as well as patient responses to therapy. It is in this regard that the *Cis*-p53TKGFP reporter system [80] might find crucial use because p53 is important for regulating a panoply of proapoptotic, repair and cell-cycle regulatory genes, including *p21/WAF1*, *MDM2*, *BCL-1* and *BAX* (see Ref. [81] and references therein). Similarly, PET imaging of prostate cancer gene expression and metastasis in sentinel lymph nodes in mice employed a prostate-specific adenoviral reporter vector, AdTSTA-sr39tk, under the control of the prostate-specific antigen gene promoter. AdTSTA-sr39tk expression was detected using ^{18}F-3′-fluoro-3′-deoxy-L-thymidine or 9-(4-[^{18}F]fluoro-3-hydroxymethylbutyl) guanine. This method of non-invasive lymphoscintigraphy could find widespread use in humans [82]. There are distinct advantages inherent in PET imaging of gene expression *in vivo* because it enables single-step, non-invasive, near-real-time *in vivo* monitoring, not only of gene activity but also of the time course and tissue specificity of expression, as well as the stability of the proteins expressed by the transgene. Because there is stochastic variation in gene expression between individuals [83], it will also enable optimization of transgene dosage required for therapy at a patient-specific level and crucially, the determination of efficacy or risk-to-benefit ratios.

Targeted immuno- and radiopharmaceutical therapy

Tumor-specific surface antigens are ideal biomarkers for targeted radiotherapy. Consequently, radiopharmaceuticals are largely based on antigen–antibody, peptide hormone–receptor and substrate–transporter systems [84–88]. These exploit radionuclides, which are predominantly beta or Auger electron emitters for therapeutic effect (Table 1). The use of ^{131}I in differentiated thyroid cancer is a well-established standard of care. This treatment exploits the sodium-iodide symporter in differentiated thyroid cancer and has proved to be effective in treating the disease [78]. Another application employs radiolabeled somatostatin peptide mimetics to target tumors expressing somatostatin receptors. This therapy has been particularly effective in neuroendocrine tumors but might also be applicable to other types of cancer because of the ubiquity of somatostatin receptors. The generation of antibodies against tumor antigens by linking highly toxic radioisotopes to cancer-cell-specific monoclonal antibodies (Mabs) provides specific tools for selective killing of cancerous cells. For example, some radiolabeled antibodies have been approved for clinical use; these include ^{131}I-labeled tositumomab and ^{90}Y-labeled ibritumomab for treating non-Hodgkins's lymphoma [84,85]. Radiolabeled Mabs against tumor-specific antigens, such as Her2 in breast cancer or the carcinoembryonic antigen for cancers of the gastrointestinal tract, have also been developed [88]. Similarly and most encouragingly, radiolabeled Mabs have also been developed against infectious diseases, including viral (e.g. HIV-1), bacterial (e.g. *Streptococcus pneumoniae*) and

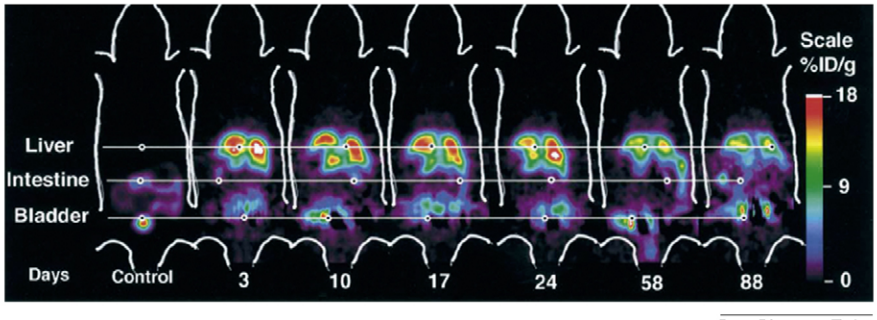

FIGURE 3

Real-time monitoring of *in vivo* gene expression by micro-PET. Sequential micro-PET images of Swiss Webster mice injected with adenoviruses expressing the herpes simplex virus 1 thymidine kinase (TK) gene under the control of the cytomegalovirus promoter. TK expression was determined over time by injecting the mice with the substrate analog for the enzyme, 9-(4-[^{18}F]-fluoro-3-hydroxymethylbutyl)guanine. Adapted, with permission, from Ref. [46].

TABLE 1

Therapeutic radionuclides in current use, with examples of isotope-liganded biomarkers (e.g. monoclonal antibodies, peptides, drugs and substrates) and relevant disease categories

No Radionuclide	Emission type	Half-life	Imageability	Liganded biomarker/clinical application
^{225}Ac	α, β	10 days	Yes	Mab/neuroendocrine tumors
^{211}At	α	7.2 hours	Yes	Mab/gliomas
^{212}Bi	α	1.0 hours	Yes	Dodecanetetraacetic acid-Mab/leukaemia
^{213}Bi	α	45.7 min	Yes	Anti-CD33 Mab/leukemia
^{67}Cu	β, γ	2.6 days	Yes	Mabs/non-Hodgkin's lymphoma
^{67}Ga	Auger, β, γ	3.3 days	Yes	Citrate/infection and inflammation
^{125}I	Auger	60.1 days	Yes	Mab/thyroid cancer
^{131}I	β, γ	8.0 days	Yes	Mab/non-Hodgkin's lymphoma; iodide/thyroid cancer; lipiodol/hepatocellular carcinoma
^{177}Lu	β, γ	6.7 days	Yes	Peptides/neuroendocrine tumors
^{186}Re	β, γ	3.8 days	Yes	Bisphosphates/bone metastasis and osteosarcoma
^{188}Re	β, γ	17.0 hours	Yes	Lipiodol/hepatocellular carcinoma
^{195m}Pt	Auger	4.0 days	No	Drugs (e.g. cisplastin)/solid tumors
^{212}Pb	β	10.6 hours	Yes	Peptides/melanoma
^{153}Sm	β, γ	2.0 days	Yes	Bisphosphonates/bone metastases and osteosarcoma
^{90}Y	β	2.7 days	Yes (brehmstrahlung)	Peptides/neuroendocrine tumors; anti-CD20 MAb/non-Hodgkin's lymphoma

Modified, with permission, from Ref. [83].

fungal infections (*Cryptococcus* and *Histoplasma*). This involves tagging select radionuclides to antibodies that recognize specific cell-surface antigens found on pathogens or pathogen-infected host cells, thus ensuring targeted killing or therapeutic selectivity [89].

General limitations in isotopic biomarker application in translational medicine

In as much as isotopes are an integral part of translational medicine and disease therapy, some intractable challenges remain. For example, some isotopes have exceedingly short half-lives (e.g. the half-lives of ^{11}C and ^{213}Bi are 20 min and 45.7 min, respectively) (Table 1). This means that radiopharmaceuticals that are based on these or similar isotopes cannot be stored or transported over a distance, thus requiring that they are produced in-house or proximally to the end user, which might not always be practical. New approaches for improving the stability of such isotopes are, therefore, required. There is also a dearth of medical isotopes because of outdated nuclear reactors; this is further compounded by a fragmented supply chain for these isotopes [90]. High equipment and maintenance costs, as well as accessibility to or technical expertise in using them, are also important bottlenecks. As for all other technologies, there are also major constraints and difficulties associated with isotopic imaging. Some of the biomarkers (e.g. the EGFR) that are used for imaging certain tumors are also present in normal cells. This means that non-specific binding or uptake of isotope-laden biomarkers might present high background imaging problems. The potential for energy scatter or signal attenuation is also high. With high-energy isotopes, this might result in collateral damage to tissues surrounding the tumor. Some isotopes, such as [^{18}F]-FDG (which is used to image tumors on the basis of increased glucose uptake and metabolism), are also markedly taken up by cells or sites of high metabolic activity, such as macrophages activated during infection and inflammation, and the heart. This lack of specificity might yield false-positive imaging information, making accurate disease diagnosis difficult. This might be overcome by incorporating a parallel subtraction algorithm in imaging software for spatial refinement or increased image resolution. At present, techniques such as PET and SPECT only produce gross anatomical spatial resolution and yield an imprecise location of diseased loci; disease diagnosis at single-cell resolution might be invaluable – for example, in aiding surgery with pin-point accuracy. Furthermore, the time required for image acquisition might exceed the half-life of some isotopes (see above).

Concluding remarks and future perspectives

Biomarkers provide a crucial bridge between basic biology and clinical medicine because they have informational value that might be used for translational research. Isotopes have contributed immensely to biomarker discovery and application in clinical medicine. Regardless of the context or method by which they might be identified, biomarkers have revolutionized molecular medicine by facilitating *in vivo* diagnostic imaging, disease staging and monitoring, as well as clinical pharmacokinetic and pharmacodynamic assessment of drug dosing during therapy. Isotopes afford increased detection sensitivity and continue to be used as tracers either unconjugated or conjugated to cell ligands, drugs and substrates, to visualize functional disease pathways, and for therapy. In spite of this, only a very small proportion of the therapeutic potential of isotopes has been achieved in the clinic; much still remains to be accomplished. For example, genetically engineered improvements are constantly being made to enhance the tumoricidal effect of isotope-laden antibodies. Equally, biomarkers are sought to develop radiolabeled small-molecule ligands with improved tumor-binding or receptor occupancy and internalization for use in diagnostic imaging and/or therapeutic monitoring. An evolving and prospectful application of isotopes in this regard is the use of nano-generators. High-energy alpha particles ensconced in these nano-generators have been used in experimental tumor therapy in mice and hold great promise for clinical application [91]. Targeting specificity and delivery of these nano-generators is provided by antibodies to the cognate tumor biomarker. This method of targeted delivery cuts cost and waste and minimizes bystander effects. If combined with the imaging capability of quantum dots (Qdots) [92], it is conceptually possible to encapsulate tumor-specific biomarkers that are liganded to functionalized Qdots and caged within nano-generators, creating 'super-smart' nano-bombs for combined tumor radiotherapy

and imaging, especially for therapeutic isotopes (such as ittrium-90) that are difficult to image.

New developments for non-invasive *in vivo* imaging of inflammation, pH differentials, metabolomics and myeloid cell function including those described above are most anticipated. With regard to inflammatory disease, one difficulty is the broad-spectrum nature of these diseases and the potential for cross-talk between inflammation-inducing signals. This makes the choice of biomarker for diagnostic imaging these diseases difficult, meaning additional measures are necessary for accurate diagnosis. Several other areas have received scant attention, probably because the field is still evolving. For example, current strategies for SNP mapping to identify human genetic disease susceptibility loci are painstakingly slow and costly [93]; innovative methods are needed for rapidly biomarking such loci. New advances in metabolomics are also urgently required to diagnose metabolic diseases (inborn errors of metabolism) to direct disease management or treatment without recourse to single-gene mutation analysis.

DNA provides a huge resource for drug discovery but biomarking DNA metabolism (e.g. its replication) is inadequate. Because most somatic cells have a finite number of DNA replication cycles, a technique that would enable this to be determined would be an invaluable tool for identifying aberrant cells before disease develops. By monitoring DNA replication in real-time using isotopically labeled DNA precursors, it might be possible, for example, to track replicative senescence, a feature that is absent or defunct in tumorigenic or stem and abnormal cells [94]. Imaging DNA metabolism *in vivo* might be particularly useful for diagnosing or monitoring tumor cell growth by measuring its DNA replication rates. Encouragingly, a radiolabeled thymidine analog 2′-deoxy-2′-[^{18}F]-fluoro-β-D-arabinofuranosyl)thymine has been used to this effect and to image tumors in humans [95]. This holds some promise – for example, in tracking the body's ability to repair DNA lesions such as pyrimidine dimers *in vivo* at single-cell resolution. This would be an enormous boost in this new age of preemptive medicine.

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References

1 Rifai, N. *et al.* (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* 24, 971–983
2 Lang, L. (2004) High clinical trials attrition rate is boosting drug development costs. *Gastroenterology* 127, 1026
3 The International Human Genome Sequencing Consortium, (2004) Finishing the euchromatic sequence of the human genome. *Nature* 431, 931–945
4 Hollstein, M. *et al.* (1991) p53 mutations in human cancers. *Science* 253, 49–53
5 Doolittle, B.R. *et al.* (2001) Detection of the mutated K-Ras biomarker in colorectal carcinoma. *Exp. Mol. Pathol.* 70, 289–301
6 Maheswaran, S. *et al.* (2008) Detection of mutations in EGFR in circulating lung-cancer cells. *N. Engl. J. Med.* 359, 366–377
7 Schena, M. *et al.* (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270, 467–470
8 Kalnina, Z. *et al.* (2005) Alterations of pre-mRNA splicing in cancer. *Genes Chromosomes Cancer* 42, 342–357
9 Chen, Y.R. *et al.* (2006) Quantitative proteomic and genomic profiling reveals metastasis-related protein expression patterns in gastric cancer cells. *J. Proteome Res.* 5, 2727–2742
10 Kirmiz, C. *et al.* (2007) A serum glycomics approach to breast cancer biomarkers. *Mol. Cell. Proteomics* 6, 43–55
11 Kyselova, Z. *et al.* (2007) Alterations in the serum glycome due to metastatic prostate cancer. *J. Proteome Res.* 6, 1822–1832
12 Barrabés, S. *et al.* (2007) Glycosylation of serum ribonuclease 1 indicates a major endothelial origin and reveals an increase in core fucosylation in pancreatic cancer. *Glycobiology* 17, 388–400
13 Ueda, K. *et al.* (2007) Comparative profiling of serum glycoproteome by sequential purification of glycoproteins and 2-nitrobenzensulfenyl (NBS) stable isotope labeling: a new approach for the novel biomarker discovery for cancer. *J. Proteome Res.* 6, 3475–3483
14 Veenstra, T.D. (2007) Global and targeted quantitative proteomics for biomarker discovery. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* 847, 3–11
15 Anderson, N.L. and Anderson, N.G. (2002) The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* 1, 845–867
16 Chaerkady, R. and Pandey, A. (2008) Applications of proteomics to lab diagnosis. *Annu. Rev. Pathol.* 3, 485–498
17 Ong, S.E. *et al.* (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 1, 376–386
18 Krüger, M. *et al.* (2008) SILAC mouse for quantitative proteomics uncovers kindlin-3 as an essential factor for red blood cell function. *Cell* 134, 353–364
19 Wu, C.C. *et al.* (2004) Metabolic labeling of mammalian organisms with stable isotopes for quantitative proteomic analysis. *Anal. Chem.* 76, 4951–4959
20 Brun, V. *et al.* (2007) Isotope-labeled protein standards: toward absolute quantitative proteomics. *Mol. Cell. Proteomics* 6, 2139–2149
21 Tannu, N.S. and Hemby, S.E. (2006) Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. *Nat. Protocols* 1, 1732–1742
22 Monribot-Espagne, C. and Boucherie, H. (2002) Differential gel exposure, a new methodology for the two-dimensional comparison of protein samples. *Proteomics* 2, 229–240
23 Poznanović, S. *et al.* (2005) Differential radioactive proteomic analysis of microdissected renal cell carcinoma tissue by 54 cm isoelectric focusing in serial immobilized pH gradient gels. *J. Proteome Res.* 4, 2117–2125
24 Seferovic, M.D. *et al.* (2008) Quantitative 2-D gel electrophoresis-based expression proteomics of albumin and IgG immunodepleted plasma. *J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci.* 865, 147–152
25 Nanavati, D. *et al.* (2008) Stoichiometry and absolute quantification of proteins with mass spectrometry using fluorescent and isotope-labeled concatenated peptide standards. *Mol. Cell. Proteomics* 7, 442–447
26 Gygi, S.P. *et al.* (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 17, 994–999
27 Tao, W.A. and Aebersold, R. (2003) Advances in quantitative proteomics via stable isotope tagging and mass spectrometry. *Curr. Opin. Biotechnol.* 14, 110–118
28 Schneider, L.V. and Hall, M.P. (2005) Stable isotope methods for high-precision proteomics. *Drug Discov. Today* 10, 353–363
29 Keshishian, H. *et al.* (2007) Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. *Mol. Cell. Proteomics* 6, 2212–2229
30 Ross, P.L. *et al.* (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 3, 1154–1169
31 Gerber, S.A. *et al.* (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. U. S. A.* 100, 6940–6945
32 Schnolzer, M. *et al.* (1996) Protease-catalyzed incorporation of ^{18}O into peptide fragments and its application for protein sequencing by electrospray and matrix-assisted laser desorption/ionization mass spectrometry. *Electrophoresis* 17, 945–953
33 Hood, B.L. *et al.* (2005) Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol. Cell. Proteomics* 4, 1741–1753
34 Stockwin, L.H. *et al.* (2006) Proteomic analysis of plasma membrane from hypoxia-adapted malignant melanoma. *J. Proteome Res.* 5, 2996–3007
35 Hall, M.P. and Schneider, L.V. (2004) Isotope-differentiated binding energy shift tags (IDBEST) for improved targeted biomarker discovery and validation. *Expert Rev. Proteomics* 1, 421–431
36 O'Hara, R.M. *et al.* (2006) Cell-surface and cytokine biomarkers in autoimmune and inflammatory disease. *Drug Discov. Today* 11, 342–347

37 Kuhn, E. *et al.* (2004) Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ^{13}C-labeled peptide standards. *Proteomics* 4, 1175–1186

38 Henley, S.M. *et al.* (2005) Biomarkers for neurodegenerative diseases. *Curr. Opin. Neurol.* 18, 698–705

39 Cassell, G.H. (1998) Infectious causes of chronic inflammatory diseases and cancer. *Emerg. Infect. Dis.* 4, 475–487

40 Censini, S. *et al.* (1996) cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. U. S. A.* 93, 14648–14653

41 Goodman, K.J. *et al.* (2005) Dynamics of *Helicobacter pylori* infection in a US-Mexico cohort during the first two years of life. *Int. J. Epidemiol.* 34, 1348–1355

42 Colina, R. *et al.* (2008) Translational control of the innate immune response through IRF-7. *Nature* 452, 323–328

43 Stojdl, D.F. *et al.* (2000) The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. *J. Virol.* 74, 9580–9585

44 Schoenheimer, R. and Rittenberg, D. (1938) The application of isotopes to the study of intermediary metabolism. *Science* 87, 221–226

45 Taylor, E.W. *et al.* (2002) Accelerating the drug optimization process: identification, structure elucidation, and quantification of *in vivo* metabolites using stable isotopes with LC/MS^n and the chemiluminescent nitrogen detector. *Anal. Chem.* 74, 3232–3236

46 Liang, Q. *et al.* (2002) Monitoring adenoviral DNA delivery, using a mutant herpes simplex virus type 1 thymidine kinase gene as a PET reporter gene. *Gene Ther.* 9, 1659–1666

47 Gallagher, F.A. *et al.* (2008) Magnetic resonance imaging of pH *in vivo* using hyperpolarized ^{13}C-labeled bicarbonate. *Nature* 453, 940–943

48 Leichert, L.I. *et al.* (2008) Quantifying changes in the thiol redox proteome upon oxidative stress *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* 105, 8197–8202

49 Butterfield, D.A. *et al.* (2006) Redox proteomics in some age-related neurodegenerative disorders or models thereof. *NeuroRx* 3, 344–357

50 Zhang, J. and Goodlett, D.R. (2004) Proteomic approach to studying Parkinson's disease. *Mol. Neurobiol.* 29, 271–288

51 Hansel, B. *et al.* (2004) Metabolic syndrome is associated with elevated oxidative stress and dysfunctional dense high-density lipoprotein particles displaying impaired antioxidative activity. *J. Clin. Endocrinol. Metab.* 89, 4963–4971

52 Van Guilder, G.P. *et al.* (2006) Influence of metabolic syndrome on biomarkers of oxidative stress and inflammation in obese adults. *Obesity (Silver Spring)* 14, 2127–2131

53 Phelps, M.E. (2000) Positron emission tomography provides molecular imaging of biological processes. *Proc. Natl. Acad. Sci. U. S. A.* 97, 9226–9233

54 Weber, W.A. *et al.* (2008) Technology insight: novel imaging of molecular targets is an emerging area crucial to the development of targeted drugs. *Nat. Clin. Pract. Oncol.* 5, 44–54

55 Mishani, E. *et al.* (2005) High-affinity epidermal growth factor receptor (EGFR) irreversible inhibitors with diminished chemical reactivities as positron emission tomography (PET)-imaging agent candidates of EGFR overexpressing tumors. *J. Med. Chem.* 48, 5337–5348

56 Wang, J.Q. *et al.* (2006) Synthesis of [^{11}C]Iressa as a new potential PET cancer imaging agent for epidermal growth factor receptor tyrosine kinase. *Bioorg. Med. Chem. Lett.* 16, 4102–4106

57 Piert, M. *et al.* (2005) Hypoxia-specific tumor imaging with ^{18}F-fluoroazomycin arabinoside. *J. Nucl. Med.* 46, 106–113

58 Beer, A.J. *et al.* (2008) Patterns of alphavbeta3 expression in primary and metastatic human breast cancer as shown by ^{18}F-Galacto-RGD PET. *J. Nucl. Med.* 49, 255–259

59 Backer, M.V. *et al.* (2007) Molecular imaging of VEGF receptors in angiogenic vasculature with single-chain VEGF-based probes. *Nat. Med.* 13, 504–509

60 Sarda-Mantel, L. *et al.* (2006) ^{99m}Tc-Annexin-V functional imaging of luminal thrombus activity in abdominal aortic aneurysms. *Arterioscler. Thromb. Vasc. Biol.* 26, 2153–2159

61 Buck, A.K. *et al.* (2003) Imaging proliferation in lung tumors with PET: ^{18}F-FLT versus ^{18}F-FDG. *J. Nucl. Med.* 44, 1426–1431

62 Judenhofer, M.S. *et al.* (2008) Simultaneous PET-MRI: a new approach for functional and morphological imaging. *Nat. Med.* 14, 459–465

63 Weissleder, R. and Pittet, M.J. (2008) Imaging in the era of molecular oncology. *Nature* 452, 580–589

64 Jaffer, F.A. *et al.* (2007) Molecular imaging of cardiovascular disease. *Circulation* 116, 1052–1061

65 Kirik, D. *et al.* (2005) Imaging in cell-based therapy for neurodegenerative diseases. *Eur. J. Nucl. Med. Mol. Imaging* 32 (Suppl 2), S417–S434

66 Radu, C.G. *et al.* (2008) Molecular imaging of lymphoid organs and immune activation by positron emission tomography with a new [(18)F]-labeled 2′-deoxycytidine analog. *Nat. Med.* 14, 783–788

67 Day, S.E. *et al.* (2007) Detecting tumor response to treatment using hyperpolarized ^{13}C magnetic resonance imaging and spectroscopy. *Nat. Med.* 13, 1382–1387

68 Davenport, A.P. and Kuc, R.E. (2005) Radioligand-binding and molecular-imaging techniques for the quantitative analysis of established and emerging orphan receptor systems. *Methods Mol. Biol.* 306, 93–120

69 Harris, D.M. *et al.* (2008) GPCR signalling in hypertension: role of GRKs. *Clin. Sci.* 115, 79–89

70 Sakurai, T. *et al.* (1998) Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92, 573–585

71 Blagoev, B. *et al.* (2003) Proteomics strategy to elucidate functional protein–protein interactions applied to EGF signaling. *Nat. Biotechnol.* 21, 315–318

72 Ray, P. *et al.* (2002) Noninvasive quantitative imaging of protein–protein interactions in living subjects. *Proc. Natl. Acad. Sci. U. S. A.* 99, 3105–3110

73 Doubrovin, M. *et al.* (2001) Imaging transcriptional regulation of p53-dependent genes with positron emission tomography *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* 98, 9300–9305

74 MacLaren, D.C. *et al.* (1999) Repetitive non-invasive imaging of the dopamine D_2 receptor as a reporter gene in living animals. *Gene Ther.* 6, 785–791

75 Zinn, K.R. *et al.* (2000) Noninvasive monitoring of gene transfer using a reporter receptor imaged with a high-affinity peptide radiolabeled with ^{99m}Tc or ^{188}Re. *J. Nucl. Med.* 41, 887–895

76 Haberkorn, U. *et al.* (2002) Functional genomics and proteomics – the role of nuclear medicine. *Eur. J. Nucl. Med. Mol. Imaging* 29, 115–132

77 Barton, K.N. *et al.* (2003) GENIS: gene expression of sodium iodide symporter for noninvasive imaging of gene therapy vectors and quantification of gene expression *in vivo*. *Mol. Ther.* 8, 508–518

78 Peñuelas, I. *et al.* (2005) Positron emission tomography imaging of adenoviral-mediated transgene expression in liver cancer patients. *Gastroenterology* 128, 1787–1795

79 Riley, T. *et al.* (2008) Transcriptional control of human p53-regulated genes. *Nat. Rev. Mol. Cell Biol.* 9, 402–412

80 Yu, Y. *et al.* (2000) Quantification of target gene expression by imaging reporter gene expression in living animals. *Nat. Med.* 6, 933–937

81 Burton, J.B. *et al.* (2008) Adenovirus-mediated gene expression imaging to directly detect sentinel lymph node metastasis of prostate cancer. *Nat. Med.* 14, 882–888

82 Bayele, H.K. and Srai, S.K. (2009) Regulatory variation in hepcidin expression as a heritable quantitative trait. *Biochem. Biophys. Res. Commun.* 384, 22–27

83 Milenic, D.E. *et al.* (2004) Antibody-targeted radiation cancer therapy. *Nat. Rev. Drug Discov.* 3, 488–499

84 Reichert, J.M. and Valge-Archer, V.E. (2007) Development trends for monoclonal antibody cancer therapeutics. *Nat. Rev. Drug Discov.* 6, 349–356

85 Adams, G.P. and Weiner, L.M. (2005) Monoclonal antibody therapy of cancer. *Nat. Biotechnol.* 23, 1147–1157

86 Wu, A.M. and Senter, P.D. (2005) Arming antibodies: prospects and challenges for immunoconjugates. *Nat. Biotechnol.* 23, 1137–1146

87 von Mehren, M. *et al.* (2003) Monoclonal antibody therapy of cancer. *Annu. Rev. Med.* 54, 343–369

88 Li, L. *et al.* (2008) A versatile bifunctional chelate for radiolabeling humanized anti-CEA antibody with In-111 and Cu-64 at either thiol or amino groups: PET imaging of CEA-positive tumors with whole antibodies. *Bioconjug. Chem.* 19, 89–96

89 Dadachova, E. and Casadevall, A. (2009) Radioimmunotherapy of infectious diseases. *Semin. Nucl. Med.* 39, 146–153

90 Gould, P. (2009) Medical isotope shortage reaches crisis level. *Nature* 460, 312–313

91 McDevitt, M.R. *et al.* (2001) Tumor therapy with targeted atomic nanogenerators. *Science* 294, 1537–1540

92 Gao, X. *et al.* (2004) *In vivo* cancer targeting and imaging with semiconductor quantum dots. *Nat. Biotechnol.* 22, 969–976

93 Campisi, J. (1997) The biology of replicative senescence. *Eur. J. Cancer* 33, 703–709

94 Peltonen, L. and McKusick, V.A. (2001) Dissecting human disease in the postgenomic era. *Science* 291, 1224–1229

95 Sun, H. *et al.* (2005) Imaging DNA synthesis with [^{18}F]FMAU and positron emission tomography in patients with cancer. *Eur. J. Nucl. Med. Mol. Imaging* 32, 15–22