Published in final edited form as:
Expert Rev Proteomics. 2012 June ; 9(3): 337–345. doi:10.1586/epr.12.21.

Advances in the proteomic investigation of the cell secretome

Kristy J Brown[1,‡], **Catherine A Formolo**[1], **Haeri Seol**[1], **Ramya L Marathi**[1], **Stephanie Duguez**[1], **Eunkyung An**[2], **Dinesh Pillai**[1], **Javad Nazarian**[1], **Brian R Rood**[3], and **Yetrib Hathout**[*,1,‡]

[1]Children's National Medical Center, Center for Genetic Medicine Research, 111 Michigan Avenue NW, Washington DC, 20010, USA

[2]Laboratory of Systems Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892, USA

[3]Children's National Medical Center, Center for Cancer and Blood Disorders, 111 Michigan Avenue NW, Washington DC, USA

Abstract

Studies of the cell secretome have greatly increased in recent years owing to improvements in proteomic platforms, mass spectrometry instrumentation and to the increased interaction between analytical chemists, biologists and clinicians. Several secretome studies have been implemented in different areas of research, leading to the generation of a valuable secretome catalogs. Secreted proteins continue to be an important source of biomarkers and therapeutic target discovery and are equally valuable in the field of microbiology. Several discoveries have been achieved *in vitro* using cell culture systems, *ex vivo* using human tissue specimens and *in vivo* using animal models. In this review, some of the most recent advances in secretome studies and the fields that have benefited the most from this evolving technology are highlighted.

Keywords

biomarkers; cancer; cerebrospinal fluid; exosomes; *in vivo* microdialysis; mass spectrometry; secretome; serum; SILAC; urine

Major advances in the proteomic investigation of the cell secretome have occurred over the last 5 years. Thanks to improvements in mass spectrometry and proteomic techniques, several in-depth secretome profiles from different cell types, body fluids and disease conditions have been established and continue to be generated at a fast pace. In the past few years, there has been an increased interest in cell and microorganism secretomes owing to the pivotal role of secreted proteins in disease biology and progression [1–5] and plant protection [6, 7] as well as in industry [8]. However, in the biomedical sector especially, the hunt for clinically meaningful targets from the cell secretome is still at the discovery phase and will require thorough data analyses and validation before transitioning into clinical practice.

[*]Author for correspondence: Tel.: +1 202 476 3136, Fax: +1 202 476 6014, yhathout@cnmcresearch.org.
[‡]Authors contributed equally.

Financial & competing interests disclosure
The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

Most secretome studies to date are performed *in vitro* on cultured cells, which may not directly correlate to the *in vivo* environment. Additionally, the requirement of incubating mammalian cells in serum-free medium to avoid interference from serum contaminants might further adulterate the cell secretome profile, reducing the correlation to the true physiological secretome. Indeed, depending on the cell type studied, the length of incubation in serum-free medium can vary from 12 to 24 h, and this might dramatically influence the secretome profiles. Some cells such as cancer cell lines are very tolerant to serumfree medium while other cells, such as primary cells, are sensitive to serum-free medium and might result in increased cell death and release of intracellular proteins. Only a few studies have attempted to survey the secretome *in vivo* or *ex vivo*, either by analyzing the secretome of tissue explants without isolating individual cells [9], or by using microdialysis devices [10–14]. The most recent advances in cell secretome studies and their applications are outlined in this review.

Secretome studies of microorganisms

Bacteria, yeast and fungi secretomes are perhaps among the easiest secretomes to study since these microorganisms do not require any serum or other exogenous protein additives in their culture media. Thus, conditioned media collected from these microorganisms contain mainly proteins released by the microorganism itself. In the past few years, there has been an increasing interest in studying the secretomes of different microorganisms owing to the roles they play in infectious diseases, the food industry and biotechnology as well as agriculture and environment. Thus, in the past few years, secretomes have been established for opportunistic microorganisms and have begun shedding light on new mechanisms of pathogenicity exerted on their hosts [15–17]. Secretomes were also established for several beneficial microorganisms such as the penicillin-producing fungi *Penicillium chrysogenum* for the pharmaceutical sector [18], as well as for yeast [19, 20] and fungi for the food industry [18]. The biotechnology industry has taken an interest in the secretomes of microorganisms that might help produce biofuels [21]. Thus efforts in the study of microorganismal secretomes may eventually lead to a wide array of discoveries, from the characterization of novel vaccines and new therapeutic strategies to the development of new energy sources.

Secretome studies of mammalian cells

Most secretome studies of mammalian cells are performed *in vitro* by first culturing cells of interest in serum-supplemented medium to obtain a sufficient number of cells. Cells are then carefully washed with sterile phosphate-buffered saline (up to six times) to remove bovine serum proteins, before incubating them in serum-free medium for another 12–24 h. Conditioned medium containing cell-secreted proteins is collected and processed for mass spectrometry analysis and protein identification (Figure 1).

In general, depending on the proteomic technique and the mass spectrometer, most mammalian cells have been found to release 100–600 proteins or more, including both classically and nonclassically secreted proteins. In the past 5 years, several secretome catalogs have been established from different cell types and conditions (Figure 2).

An interesting observation from these *in vitro* secretome studies is the fact that several intracellular proteins are consistently found to be released by cultured mammalian cells into the conditioned medium. Although these intracellular proteins can be released due to cell death or leakage, there is now evidence that some of these proteins are secreted via nonclassical pathways, perhaps via vesicles and exosomes [22], and may have extracellular functions. For example, α-enolase (ENOA), a glycolytic enzyme involved in the synthesis of pyruvate, is consistently detected in significant amounts in the secretome of almost every

type of cultured mammalian cell. Extracellular ENOA or cell surface ENOA was suggested to act as a plasminogen receptor mediating extracellular matrix degradation and cell migration in cancer and to have diagnostic and prognostic value [23]. Also, this protein was consistently detected in an exosome preparation of hepatocytes [24].

Perhaps the biggest challenge in secretomic studies lies in discriminating proteins that are truly secreted from those that are released as the result of nonphysiological stresses of mechanical injury. To minimize these issues, a few techniques have been proposed, such as the hollow-fiber culture system, which enables high-density cell culture with minimal cell lysis [25]. This technique allowed up to 61% enrichment of true signal peptide-containing proteins. However, there were still 36% intracellular proteins detected in the secretome using this method, and ENOA was again abundant in the conditioned medium. This suggests that there are nonclassical mechanisms by which these intracellular proteins are released by cultured cells. Indeed, a recent review has proposed several mechanisms by which some intracellular proteins are exported to the extracellular compartment and that these proteins usually perform additional extracellular functions that are different from their intracellular role [26].

Protein secretion via exosomes

Exosome shedding is another mechanism by which some intracellular proteins are secreted by mammalian cells. Exosomes are small vesicles with an average size ranging from 30 to 150 nm. They are believed to originate from endosomes and contain a variety of components including mRNA, miRNA and proteins such as carbohydrate and lipid metabolism enzymes, cytoskeletal components and chaperone proteins [27–30]. Exosomes are becoming increasingly targeted in the f ield of biomedical sciences as they seem to be involved in a number of biological and physiological functions [28, 29]. Exosomes are not only detected *in vitro* in the conditioned medium of cultured mammalian cells but are also found circulating *in vivo* in blood [31], urine [32] and even in cerebrospinal fluid [33]. The exact role of exosomes is not well understood at this point, but it has been suggested that they might play a role in protein turnover, serve as carriers for RNA and miRNA as well as play a role in the immune system [34]. In brief, several studies have investigated and continue to investigate the proteome of exosomes and their potential diagnostic and therapeutic value [35].

Secretome application studies

Secretome in cancer research

Perhaps one of the biomedical fields that has used secretome technology the most is oncology. Identification of novel cancer-specific biomarkers directly from serum and other body fluids using a proteomic approach has been challenging. These specimens are inherently complex with a wide dynamic range of protein concentrations, making detection of low abundant biomarkers difficult. Hence, efforts have been turned toward the analysis of cell secretomes in the hope of identifying reliable and useful cancer markers that can then be traced back in serological samples using more sensitive and targeted analyses such as ELISA, western blot or multiple-reaction monitoring mass spectrometry. A number of secretome catalogs of different cancer cell lines have been published in the last 5 years (Figure 3). These include pancreatic cancer [36, 37], breast cancer [38], ovarian cancer [39], bladder cancer [40], colorectal cancer [41], prostate cancer [42], gastric cancer [43], thyroid cancer [44], lung cancer [45, 46], head and neck cancer [47], and oral cancer [48]. In brief, among the approximately 500 articles on secretome, more than 150 are related to cancer, with those on breast cancer cells and lung cancer cells accounting for more than 25% of all cancer secretome studies (Figure 3).

Overall, *in vitro* secretome studies in cancer have generated large data sets that await careful data mining and validation in order to identify reliable and useful biomarkers associated with cancer. Several reviews have documented the utility of studying the secretome of cancer cells [4, 49, 50]. The secretome catalogs of all cancer cell lines studied could be easily deposited in a public domain database and would be a valuable tool for data mining and validation of cancer-specific markers. For instance, Wu *et al.* surveyed the secretomes of 23 cancer cell lines using a label-free proteome profiling strategy and identified 4584 proteins in the conditioned medium of all the cell lines combined [5]. From this study, they identified pan-cancer biomarkers secreted by most cancer cell lines as well as cancer type-specific biomarkers [5]. As a further example, chitinase-3-like protein 1, also known as YKL-40, was found to be secreted by several types of aggressive solid tumors [51] including glioblastoma multiforme, an aggressive type of brain tumor [2]. This protein is also detected in tissue and serum samples from cancer patients and is associated with poor prognosis [51]. Furthermore, this same protein positively correlated with the infiltrative nature of glioblastoma and other tumors, suggesting a potential role in tumor cell invasion. However, the usefulness of this protein as a valid biomarker or a therapeutic target still awaits validation. Nevertheless, this is one example of the many proteins identified using *in vitro* cell secretome analysis that may have prognostic and therapeutic value. Perhaps one day secretome profiling efforts will lead to a breakthrough in the field of oncology.

Secretome in metabolic disorders

Diseases such as Type 2 diabetes, hypertension and obesity are prevalent disorders affecting as much as one-half of the population in developed countries [52]. It is believed that adipose and skeletal tissue play major roles in these disorders. Recent studies have surveyed the secretomes of adipose cells [53], skeletal muscle cells [54] and endothelial cells [55]. Again, several proteins were cataloged from these different cell types. However, interesting proteins such as adipokines and myokines were still difficult to detect using proteomics-based approaches, probably due to their low abundance, short half-lives or low molecular masses.

In vitro secretome profiling is a valuable and simple strategy to characterize and quantify secreted proteins by a given cell under specific conditions. Whether these same proteins are secreted *in vivo*, especially considering that secretome studies are performed on cells grown in serum-free medium, remains to be examined. One study by Colzani *et al.* attempted to characterize the secretome of breast cancer cell lines in the presence of 10% bovine serum using the stable isotope labeling by amino acids in cell culture strategy in combination with ProteoMiner technology (the ProteoMiner technology uses a negative isolation of highly abundant proteins to reduce the dynamic range and allow detection of low abundant proteins [56]). This enabled detection of breast cancer cell secreted proteins under normal culture conditions (e.g., in the presence of 10% bovine serum). However, comparison between the secretomes from cells grown in serum-supplemented medium and those grown in serum-free medium was not performed in this study, and thus the true effect of serum on the cell secretome could not be evaluated.

Ex vivo & *in vivo* secretome profiling

Analyzing secreted proteins from tissue explants has been challenging owing to the low abundance of secreted proteins relative to total proteins in tissue and the difficulty of accessing the extracellular milieu in a tissue section. Additionally, most tissues are composed of a mixture of different cell types including the cells of interest, endothelial cells and fibroblast cells, making it difficult to assess cell-type specific secretomes *ex vivo* or *in vivo*. A few strategies have been developed to probe the secretome under physiological conditions, and these are outlined later in the text with their advantages and disadvantages.

Ex vivo secretome profiling from tissue explants

This strategy consists of incubating freshly obtained tissue specimens in serum-free basal medium for 12–24 h followed by collection and processing of the conditioned medium for proteome profiling. This strategy was recently used to study secretomes of equine and human cartilage explants [57, 58] and human adipose tissue [59, 60], resulting in the identification of 56 and 213 proteins, respectively. Analysis of the tissue secretome without intermediate cell culture could mimic physiological conditions because cells are still in their physiological environment interacting with other cells that might secrete factors that modulate their secretome. However, to perform such a study, careful sample handling and culturing is required to avoid false-positive discoveries due to serum proteins remaining in the tissue or proteins released by dying cells. To minimize such a confounding background, and especially blood-contaminant proteins that are often present in high abundance, tissue samples need to be extensively washed and/or perfused with sterile phosphate-buffered saline solutions to remove excess blood before incubation in serum-free medium. Roelofsen *et al.* performed an exquisite strategy enabling identification and quantification of genuine adipose tissue-secreted proteins in the presence or absence of insulin [60]. In this study, the authors partially labeled *de novo* synthesized protein *in situ* by incubating tissue sections in the medium where $^{12}C_6$-Lys was replaced by the heavy isotope $^{13}C_6$-Lys for a short period of time (up to 100 culture hours) with frequent medium changes in the first 24 h to allow for depletion of residual endogenous Lys, followed by an additional 72 h to allow sufficient incorporation of $^{13}C_6$-Lys into *de novo* synthesized proteins. Using this strategy, the authors were able to unambiguously identify 213 and 247 proteins that were newly synthesized and secreted into the extracellular milieu by adipose tissue with and without insulin treatment, respectively. Of these proteins, more than 70% were potentially secreted while the rest were of intracellular origin similar to those detected in cell culture systems. However, the rate of incorporation of $^{13}C_6$-Lys was lower in intracellular proteins compared with secreted proteins, perhaps due to the premature release of some intracellular proteins from dying cells during the tissue culture or the slow turnover of intracellular proteins relative to extracellular proteins. Interestingly, in this study, the authors identified several secreted proteins with selectively higher labeling efficiency during the 72-h incubation period. These included proteins with a high turnover rate such as chemokines and complement components. Metabolic labeling of whole tissue does not appear to require 100% labeling as in stable isotope labeling by amino acids in cell culture but rather partial labeling that enables differential protein secretion profiling between control and experimental conditions. However, the tissue specimens to be compared must be histologically similar and contain the same percentage of different cell types. For this reason, Roelofsen *et al.* performed their experiment in triplicate [60].

In vivo secretome profiling

Logically, the *in vivo* secretome in mammals could be accessed via the analysis of body fluids including serum, cerebrospinal fluid, urine, amniotic fluid, synovial fluid, pleural effusion, bronchial washes, ear fluids, saliva, tears and interstitial fluid [61–66]. A number of studies have investigated the proteomes of some of these body fluids in healthy and disease conditions to gain knowledge about their protein content. However, challenges were encountered during analysis including dynamic range (affecting detection of low abundance biomarkers), difficulty assessing the relative contribution of a specific tissue to the proteome of a specific body fluid and the possibility of contamination by blood. To circumvent some of these confounding backgrounds when studying body fluids to mine for tissue-specific biomarkers, one can compare the proteome of a given body fluid with the secretome of cells originating from the tissue in proximity to this body fluid, such as previously documented in a combined analysis of the secretome of glial cells and the cerebrospinal fluid proteome [67].

Perhaps one of the most interesting and challenging body fluids to access is the interstitial fluid, containing proteins that are secreted locally by tissues and cells in their native microenvironment. Proteins from the interstitial fluid cannot be identified by studying circulatory fluids, but a few studies have attempted to capture the interstitial fluid at its site of origin using *in vivo* microdialysis devices [10–14, 64] or a capillary ultrafiltration (CUF) probe [68]. *In vivo* microdialysis is a versatile technique widely used in neuroscience that has broad clinical and translational applications (for more details, see review Korf *et al.* [69]). However, in the context of proteomics, the technique seems to work best for small peptides and not very well for large proteins because small peptides can easily traverse the dialysis membrane while large proteins need additional time. For this reason, capillary ultrafiltration [68] and ultraslow novel microdialysis devices have been developed to capture larger proteins [69, 70]. In general, the slower the flow rate inside the dialysis tubing the better the recovery of circulating proteins/peptides [71]. This microdialysis technique seems quite promising and suitable for studying the brain secretome under healthy and disease conditions, as well as those from other tissues such as adipose, muscle, liver and solid tumors.

A recent and promising technology that might also aid in the detection of secreted proteins *in vivo* is the use of peptide-based hydrogel vesicles. These are injectable peptides that form extensive nanofiber networks resulting in a viscoelastic hydrogel. These hydrogels can capture secreted proteins from cultured cells. The technique was used as a delivery agent for growth factors and might be a useful tool to capture secreted proteins *in vivo*. A challenge for this technique is to collect these nanoparticles after injection.

Expert commentary

With the advent of proteomic techniques and mass spectrometry instrumentation, the efficiency of identifying and quantifying proteins in biological samples, including secretomes, has greatly improved. Ten years ago, only specialized laboratories equipped with the most sophisticated mass spectrometry instruments were able to generate valuable proteomics data. Currently, several biology and proteomics core facilities are capable of generating reliable and accurate proteomics data. Indeed, mass spectrometry accuracy and resolution increased from 100 ppm error and 2500 resolution to better than 10 ppm error and 40,000 resolution in 10 years. These high-precision instruments have enabled the generation of high-quality data. Thus, it is now feasible to compare secretome data obtained across different laboratories on specific cell/tissue types and specific diseases to validate and extract meaningful outcomes.

Studying the cell secretome at its origin rather than analyzing body fluids is more reliable in defining specific and sensitive biomarkers and might bring insight as to the role of locally secreted proteins under the biological condition studied. Indeed, relevant candidate proteins that are secreted by a given tissue might never reach circulating body fluids such as plasma and urine. For example, a protein secreted by the brain cells has to traverse the brain–blood barrier in order to enter the plasma circulation. Even if this occurs, the protein candidate will be extremely diluted and difficult to detect via direct analysis of the body fluid. Thus, the study of the cell secretome could be used as discovery phase to define reliable protein candidates, which can be traced back in accessible body fluids using a more sensitive method such as ELISA.

In general, the cell secretome has been found to be a valuable source for biomarker and therapeutic target discovery not only in the field of cancer but also in a number of other diseases including metabolic disorders, neurodegenerative diseases, pulmonary diseases and cardiovascular diseases. With the increasing number of studies and secretome catalogs,

creation of a public domain for a secretome database to query potential biomarkers based on the tissue and disease studied would be highly valuable. There is currently an initiative to deposit both raw mass spectrometry data and the corresponding list of identified proteins in public domains such as PRIDE [101] and ProteomeXchange [102] for proteomics-related studies. Such initiatives can greatly facilitate inter-laboratory data comparison since the most important details related to the raw mass spectra are also deposited. Furthermore, with improved mass spectrometry instrumentation and mass accuracy, secretome query databases can be harnessed to extract the most meaningful protein candidate(s) and enable comparisons across different studies as for mRNA microarray data through the cancer Biomedical Informatics Grid maintained by the National Cancer Institute [103]. These will also help refine secretome knowledge databases such as the one created recently for the fungal secretome [72].

Finally, it is important to point out that secretomics is a fairly new and emerging proteomics technique and is already contributing to a number of useful public areas including the food industry, biomedical, pharmaceutical, agricultural and biomedical sectors as well as the biofuel industry. Table 1 summarizes the findings from some key secretomics studies and the outcomes in these different areas. Please also refer to Supplementary Table 1 (table available at: www.expert-reviews.com/doi/suppl/10.1586/EPR.12.21).

Five-year view

The study of secreted proteins or secretomes holds great promise not only in the biomedical sector but also in the biotechnology and agricultural sectors. Since our last review, a number of studies have contributed to the establishment of hundreds of secretome catalogs for specific cells, tissues, organs, diseases and conditions. Five years from now, even more valuable data will be generated that will likely contribute to breakthroughs in the field of biomedical sciences by finding sensitive and specific biomarkers and eventually novel therapeutic targets for clinical practice. Indeed, secreted proteins can be easily accessed by pharmaceutical drugs and manipulated for treatment, more so than intracellular proteins. However, there is still a huge gap between the number of *in vitro* and *in vivo* studies in this area of research. This is probably due to the fact that cultured cells are easily manipulated to obtain an adequate amount of secreted proteins on which to perform mass spectrometry analysis. Nevertheless, with the improvement of microdialysis techniques, tissue fractionation, mass spectrometer instrument dynamic range and sensitivity, and proteomics strategies, there will be more *in vivo* secretome data generated in the near future.

It is becoming evident that investigating the role of secreted proteins in healthy and diseased conditions will help define valuable novel diagnostic and therapeutic strategies to treat morbid diseases such as cancer, diabetes and autoimmune diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partially supported by NIH core grants: NCMRR/NINDS R24HD050846 (National Center for Medical Rehabilitation Research) and IDDRC P30HD040677 (Intellectual and Developmental Disabilities Research Center) and by NIHNCRR UL1RR031988 (GWU-CNMC CTSI).

References

Papers of special interest have been highlighted as:

•• of considerable interest

1. An E, Sen S, Park SK, Gordish-Dressman H, Hathout Y. Identification of novel substrates for the serine protease HTRA1 in the human RPE secretome. Invest. Ophthalmol. Vis. Sci. 2010; 51(7): 3379–3386. [PubMed: 20207970] •• Discusses the comprehensive secretome of human retinal pigment epithelial cells.
2. Formolo CA, Williams R, Gordish-Dressman H, MacDonald TJ, Lee NH, Hathout Y. Secretome signature of invasive glioblastoma multiforme. J. Proteome Res. 2011; 10(7):3149–3159. [PubMed: 21574646] •• Discusses the comprehensive secretome of glioblastoma 'most invasive brain tumor cells'.
3. Korpal M, Ell BJ, Buffa FM, et al. Direct targeting of Sec23a by miR-200s influences cancer cell secretome and promotes metastatic colonization. Nat. Med. 2011; 17(9):1101–1108. [PubMed: 21822286] •• Discusses the role miRNA plays in tumor metastasis via secreted proteins.
4. Pavlou MP, Diamandis EP. The cancer cell secretome: a good source for discovering biomarkers? J. Proteomics. 2010; 73(10):1896–1906. [PubMed: 20394844]
5. Wu CC, Hsu CW, Chen CD, et al. Candidate serological biomarkers for cancer identified from the secretomes of 23 cancer cell lines and the human protein atlas. Mol. Cell Proteomics. 2010; 9(6): 1100–1117. [PubMed: 20124221] •• Discusses the comprehensive secretome profile of 23 cancer cell lines.
6. Agrawal GK, Jwa NS, Lebrun MH, Job D, Rakwal R. Plant secretome: unlocking secrets of the secreted proteins. Proteomics. 2010; 10(4):799–827. [PubMed: 19953550]
7. Stassen JH, Seidl MF, Vergeer PW, et al. Effector identification in the lettuce downy mildew *Bremia lactucae* by massively parallel transcriptome sequencing. Mol. Plant Pathol. 2012 (Epub ahead of print).
8. Bobadilla Fazzini RA, Levican G, Parada P. *Acidithiobacillus thiooxidans* secretome containing a newly described lipoprotein Licanantase enhances chalcopyrite bioleaching rate. Appl. Microbiol. Biotechnol. 2011; 89(3):771–780. [PubMed: 21191788] •• Defines a novel bacterial-secreted protein that can improve metal extraction from rocks.
9. Celis JE, Moreira JM, Cabezón T, et al. Identification of extracellular and intracellular signaling components of the mammary adipose tissue and its interstitial fluid in high risk breast cancer patients: toward dissecting the molecular circuitry of epithelial–adipocyte stromal cell interactions. Mol. Cell Proteomics. 2005; 4(4):492–522. [PubMed: 15695426]
10. Behrens HL, Li L. Monitoring neuropeptides *in vivo* via microdialysis and mass spectrometry. Methods Mol. Biol. 2010; 615:57–73. [PubMed: 20013200]
11. Bernay B, Gaillard MC, Guryca V, et al. Discovering new bioactive neuropeptides in the striatum secretome using *in vivo* microdialysis and versatile proteomics. Mol. Cell Proteomics. 2009; 8(5): 946–958. [PubMed: 19164277]
12. Dayon L, Turck N, Garcí-Berrocoso T, et al. Brain extracellular fluid protein changes in acute stroke patients. J. Proteome Res. 2011; 10(3):1043–1051. [PubMed: 21142207]
13. Xu BJ, Yan W, Jovanovic B, et al. Microdialysis combined with proteomics for protein identification in breast tumor microenvironment *in vivo* . Cancer Microenviron. 2010; 4(1):61–71. [PubMed: 21505562]
14. Yang S, Huang CM. Recent advances in protein profiling of tissues and tissue fluids. Expert Rev. Proteomics. 2007; 4(4):515–529. [PubMed: 17705709]
15. Hruskova-Heidingsfeldova O. Secreted proteins of *Candida albicans* . Front. Biosci. 2008; 13:7227–7242. [PubMed: 18508730]
16. Kaakoush NO, Man SM, Lamb S, et al. The secretome of *Campylobacter concisus* . FEBS J. 2010; 277(7):1606–1617. [PubMed: 20148967]
17. Mariappan V, Vellasamy KM, Thimma JS, Hashim OH, Vadivelu J. Identification of immunogenic proteins from *Burkholderia cepacia* secretome using proteomic analysis. Vaccine. 2010; 28(5):1318–1324. [PubMed: 19944788]
18. Jami MS, García-Estrada C, Barreiro C, Cuadrado AA, Salehi-Najafabadi Z, Martín JF. The *Penicillium chrysogenum* extracellular proteome. Conversion from a food-rotting strain to a

versatile cell factory for white biotechnology. Mol. Cell Proteomics. 2010; 9(12):2729–2744. [PubMed: 20823121]

19. Buerth C, Heilmann CJ, Klis FM, de Koster CG, Ernst JF, Tielker D. Growthdependent secretome of *Candida utilis* . Microbiology (Reading, Engl.). 2011; 157(Pt 9):2493–2503.
20. Madinger CL, Sharma SS, Anton BP, et al. The effect of carbon source on the secretome of *Kluyveromyces lactis* . Proteomics. 2009; 9(20):4744–4754. [PubMed: 19743416]
21. Herpoël-Gimbert I, Margeot A, Dolla A, et al. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. Biotechnol. Biofuels. 2008; 1(1): 18. [PubMed: 19105830]
22. Nickel W. Pathways of unconventional protein secretion. Curr. Opin. Biotechnol. 2010; 21(5):621–626. [PubMed: 20637599]
23. Capello M, Ferri-Borgogno S, Cappello P, Novelli F. α-enolase: a promising therapeutic and diagnostic tumor target. FEBS J. 2011; 278(7):1064–1074. [PubMed: 21261815]
24. Conde-Vancells J, Rodriguez-Suarez E, Embade N, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. J. Proteome Res. 2008; 7(12):5157–5166. [PubMed: 19367702]
25. Wu HY, Chang YH, Chang YC, Liao PC. Proteomics analysis of nasopharyngeal carcinoma cell secretome using a hollow fiber culture system and mass spectrometry. J. Proteome Res. 2009; 8(1):380–389. [PubMed: 19012429]
26. Maxwell CA, McCarthy J, Turley E. Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions? J. Cell. Sci. 2008; 121(Pt 7):925–932. [PubMed: 18354082]
27. Johnstone RM, Bianchini A, Teng K. Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. Blood. 1989; 74(5): 1844–1851. [PubMed: 2790208]
28. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. Immunol. Lett. 2006; 107(2):102–108. [PubMed: 17067686]
29. Pant S, Hilton H, Burczynski ME. The multifaceted exosome: biogenesis, role in normal and aberrant cellular function, and frontiers for pharmacological and biomarker opportunities. Biochem. Pharmacol. 2012; 83(11):1484–1494. [PubMed: 22230477]
30. Trams EG, Lauter CJ, Salem N Jr, Heine U. Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim. Biophys. Acta. 1981; 645(1):63–70. [PubMed: 6266476]
31. Skog J, Würdinger T, van Rijn S, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat. Cell Biol. 2008; 10(12): 1470–1476. [PubMed: 19011622]
32. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc. Natl Acad. Sci. USA. 2004; 101(36):13368–13373. [PubMed: 15326289]
33. Vella LJ, Greenwood DL, Cappai R, Scheerlinck JP, Hill AF. Enrichment of prion protein in exosomes derived from ovine cerebral spinal fluid. Vet. Immunol. Immunopathol. 2008; 124(3–4): 385–393. [PubMed: 18501435]
34. Mignot G, Roux S, Thery C, Ségura E, Zitvogel L. Prospects for exosomes in immunotherapy of cancer. J. Cell. Mol. Med. 2006; 10(2):376–388. [PubMed: 16796806]
35. Simpson RJ, Lim JW, Moritz RL, Mathivanan S. Exosomes: proteomic insights and diagnostic potential. Expert Rev. Proteomics. 2009; 6(3):267–283. [PubMed: 19489699]
36. Grønborg M, Kristiansen TZ, Iwahori A, et al. Biomarker discovery from pancreatic cancer secretome using a differential proteomic approach. Mol. Cell Proteomics. 2006; 5(1):157–171. [PubMed: 16215274]
37. Schiarea S, Solinas G, Allavena P, et al. Secretome analysis of multiple pancreatic cancer cell lines reveals perturbations of key functional networks. J. Proteome Res. 2010; 9(9):4376–4392. [PubMed: 20687567]
38. Kulasingam V, Diamandis EP. Proteomics analysis of conditioned media from three breast cancer cell lines: a mine for biomarkers and therapeutic targets. Mol. Cell Proteomics. 2007; 6(11):1997–2011. [PubMed: 17656355]

39. Faça VM, Hanash SM. In-depth proteomics to define the cell surface and secretome of ovarian cancer cells and processes of protein shedding. Cancer Res. 2009; 69(3):728–730. [PubMed: 19155298]
40. Makridakis M, Roubelakis MG, Bitsika V, et al. Analysis of secreted proteins for the study of bladder cancer cell aggressiveness. J. Proteome Res. 2010; 9(6):3243–3259. [PubMed: 20423150]
41. Xue H, Lü B, Zhang J, et al. Identification of serum biomarkers for colorectal cancer metastasis using a differential secretome approach. J. Proteome Res. 2010; 9(1):545–555. [PubMed: 19924834]
42. Goo YA, Goodlett DR. Advances in proteomic prostate cancer biomarker discovery. J. Proteomics. 2010; 73(10):1839–1850. [PubMed: 20398807]
43. Yan GR, Ding W, Xu SH, et al. Characterization of phosphoproteins in gastric cancer secretome. OMICS. 2011; 15(1–2):83–90. [PubMed: 20726782]
44. Kashat L, So AK, Masui O, et al. Secretome-based identification and characterization of potential biomarkers in thyroid cancer. J. Proteome Res. 2010; 9(11):5757–5769. [PubMed: 20873772]
45. Chenau J, Michelland S, de Fraipont F, et al. The cell line secretome, a suitable tool for investigating proteins released *in vivo* by tumors: application to the study of p53-modulated proteins secreted in lung cancer cells. J. Proteome Res. 2009; 8(10):4579–4591. [PubMed: 19639960]
46. Luo X, Liu Y, Wang R, Hu H, Zeng R, Chen H. A high-quality secretome of A549 cells aided the discovery of C4b-binding protein as a novel serum biomarker for non-small cell lung cancer. J. Proteomics. 2011; 74(4):528–538. [PubMed: 21262398]
47. Ralhan R, Masui O, Desouza LV, Matta A, Macha M, Siu KW. Identification of proteins secreted by head and neck cancer cell lines using LC-MS/MS: strategy for discovery of candidate serological biomarkers. Proteomics. 2011; 11(12):2363–2376. [PubMed: 21598386]
48. Yu CJ, Chang KP, Chang YJ, et al. Identification of guanylate-binding protein 1 as a potential oral cancer marker involved in cell invasion using omics-based analysis. J. Proteome Res. 2011; 10(8): 3778–3788. [PubMed: 21714544]
49. Karagiannis GS, Pavlou MP, Diamandis EP. Cancer secretomics reveal pathophysiological pathways in cancer molecular oncology. Mol. Oncol. 2010; 4(6):496–510. [PubMed: 20934395]
50. Makridakis M, Vlahou A. Secretome proteomics for discovery of cancer biomarkers. J. Proteomics. 2010; 73(12):2291–2305. [PubMed: 20637910]
51. Johansen JS, Jensen BV, Roslind A, Nielsen D, Price PA. Serum YKL-40, a new prognostic biomarker in cancer patients? Cancer Epidemiol. Biomarkers Prev. 2006; 15(2):194–202. [PubMed: 16492905]
52. Crawford AG, Cote C, Couto J, et al. Prevalence of obesity, Type II diabetes mellitus, hyperlipidemia, and hypertension in the United States: findings from the GE Centricity Electronic Medical Record database. Popul. Health Manag. 2010; 13(3):151–161. [PubMed: 20521902]
53. Kratchmarova I, Kalume DE, Blagoev B, et al. A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. Mol. Cell Proteomics. 2002; 1(3):213–222. [PubMed: 12096121]
54. Norheim F, Raastad T, Thiede B, Rustan AC, Drevon CA, Haugen F. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am J. Physiol. Endocrinol. Metab. 2011; 301(5):E1013–E1021. [PubMed: 21828336]
55. Burghoff S, Schrader J. Secretome of human endothelial cells under shear stress. J. Proteome Res. 2011; 10(3):1160–1169. [PubMed: 21184611]
56. Colzani M, Waridel P, Laurent J, Faes E, Rüegg C, Quadroni M. Metabolic labeling and protein linearization technology allow the study of proteins secreted by cultured cells in serum-containing media. J. Proteome Res. 2009; 8(10):4779–4788. [PubMed: 19711906]
57. Clutterbuck AL, Smith JR, Allaway D, Harris P, Liddell S, Mobasheri A. High throughput proteomic analysis of the secretome in an explant model of articular cartilage inflammation. J. Proteomics. 2011; 74(5):704–715. [PubMed: 21354348]
58. Polacek M, Bruun JA, Johansen O, Martinez I. Differences in the secretome of cartilage explants and cultured chondrocytes unveiled by SILAC technology. J. Orthop. Res. 2010; 28(8):1040–1049. [PubMed: 20108312]

59. Alvarez-Llamas G, Szalowska E, de Vries MP, et al. Characterization of the human visceral adipose tissue secretome. Mol. Cell Proteomics. 2007; 6(4):589–600. [PubMed: 17255083]

60. Roelofsen H, Dijkstra M, Weening D, de Vries MP, Hoek A, Vonk RJ. Comparison of isotope-labeled amino acid incorporation rates (CILAIR) provides a quantitative method to study tissue secretomes. Mol. Cell Proteomics. 2009; 8(2):316–324. [PubMed: 18840871] •• The authors identified genuinely secreted proteins from intact adipose tissue explant using stable isotope-labeled amino acid incorporation.

61. Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S. Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. OMICS. 2011; 15(6):353–361. [PubMed: 21568728]

62. Fliser D, Novak J, Thongboonkerd V, et al. Advances in urinary proteome analysis and biomarker discovery. J. Am. Soc. Nephrol. 2007; 18(4):1057–1071. [PubMed: 17329573]

63. Hu S, Loo JA, Wong DT. Human body fluid proteome analysis. Proteomics. 2006; 6(23):6326–6353. [PubMed: 17083142]

64. Preciado D, Goyal S, Rahimi M, et al. MUC5B is the predominant mucin glycoprotein in chronic otitis media fluid. Pediatr. Res. 2010; 68(3):231–236. [PubMed: 20531251]

65. Ray S, Reddy PJ, Jain R, Gollapalli K, Moiyadi A, Srivastava S. Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. Proteomics. 2011; 11(11):2139–2161. [PubMed: 21548090]

66. Romeo MJ, Espina V, Lowenthal M, Espina BH, Petricoin EF 3rd, Liotta LA. CSF proteome: a protein repository for potential biomarker identification. Expert Rev. Proteomics. 2005; 2(1):57–70. [PubMed: 15966853]

67. Suk K. Combined analysis of the glia secretome and the CSF proteome: neuroinflammation and novel biomarkers. Expert Rev. Proteomics. 2010; 7(2):263–274. [PubMed: 20377392]

68. Huang CM, Nakatsuji T, Liu YT, Shi Y. *In vivo* tumor secretion probing via ultrafiltration and tissue chamber: implication for anti-cancer drugs targeting secretome. Recent Pat. Anticancer Drug Discov. 2008; 3(1):48–54. [PubMed: 18289123]

69. Korf J, Huinink KD, Posthuma-Trumpie GA. Ultraslow microdialysis and microfiltration for in-line, on-line and off-line monitoring. Trends Biotechnol. 2010; 28(3):150–158. [PubMed: 20079945] •• Detailed review on novel ultraslow microdialysis for *in vivo* sampling of secreted metabolites and proteins.

70. Takeda S, Sato N, Ikimura K, Nishino H, Rakugi H, Morishita R. Novel microdialysis method to assess neuropeptides and large molecules in free-moving mouse. Neuroscience. 2011; 186:110–119. [PubMed: 21530615]

71. Li Q, Zubieta JK, Kennedy RT. Practical aspects of *in vivo* detection of neuropeptides by microdialysis coupled off-line to capillary LC with multistage MS. Anal. Chem. 2009; 81(6): 2242–2250. [PubMed: 19196160]

72. Lum G, Min XJ. FunSecKB: the Fungal Secretome KnowledgeBase. Database (Oxford). 2011; 2011:bar001. [PubMed: 21300622] •• Comprehensive database of the fungal secretome.

Websites

101. Proteomics Identifications database (PRIDE). www.ebi.ac.uk/pride

102. The ProteomeXchange Consortium. www.proteomexchange.org

103. Cancer Biomedical Informatics Grid. https://cabig.nci.nih.gov

104. UniProt. www.uniprot.org

105. SignalP 4.0 Server. www.cbs.dtu.dk/services/SignalP

Key issues

- Secretome or secretomics continue to be a valuable source for biomarkers and therapeutic target discovery.
- The development of new technologies to enable *in vivo* secretome profiling will greatly advance the field of secretome research in biomedical sciences.
- Many proteins that were believed to be strictly intracellular are now detected in the extracellular milieu both *in vitro* and *in vivo*. The role of these proteins is not well known at present.
- Intracellular proteins that are released in the extracellular milieu, however, remain a major issue in secretomics studies because it is not known if these are truly released via nonclassical secretion pathways or just as a result of the nonphysiological stresses of mechanical injury.
- Defining the exosomal proteome both *in vivo* and *in vitro* might enable novel discoveries of the role of these proteins in the extracellular space.

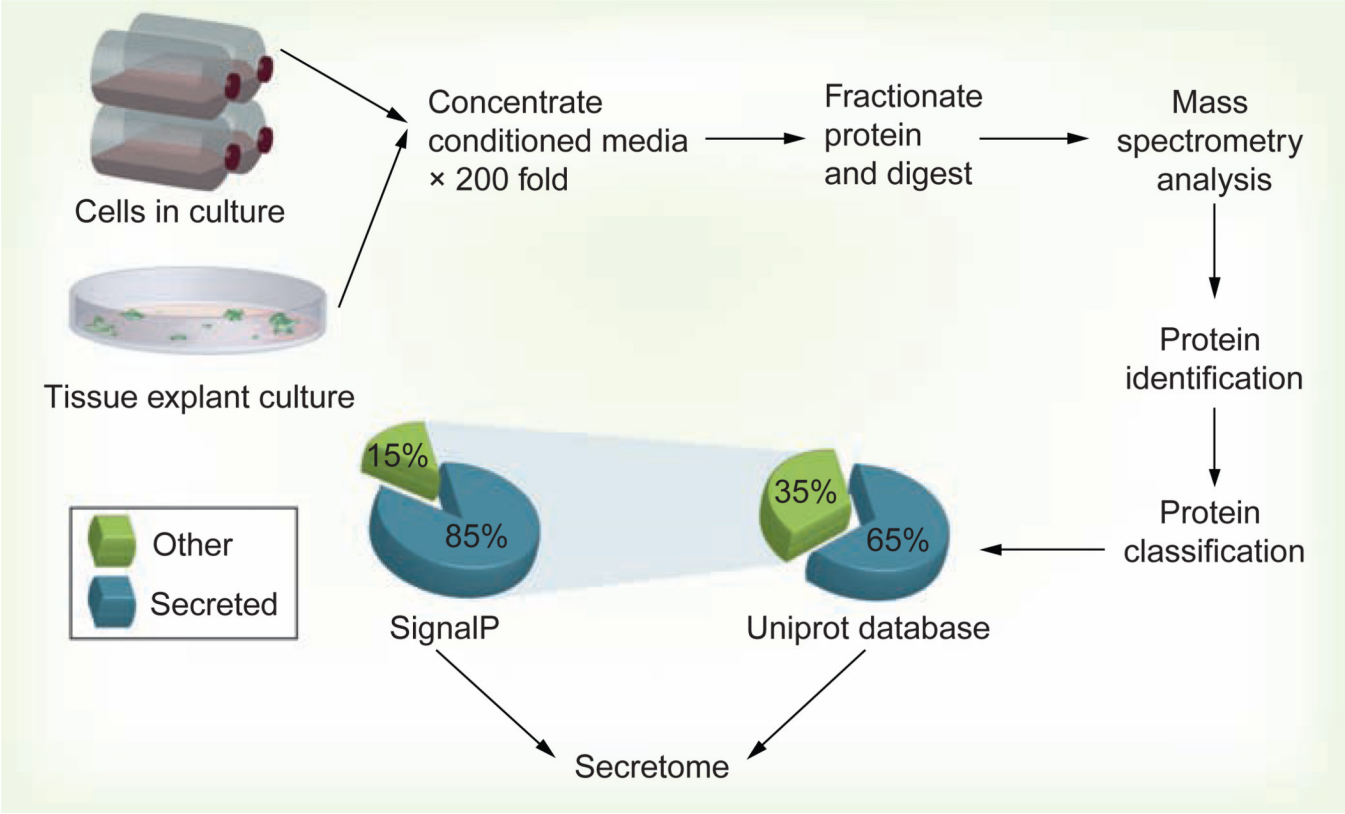

Figure 1. Generic *in vitro* secretome profiling flow chart

Typically, cells are grown to 80 or 100% confluence in serum-supplemented media, then washed (up to six times with serum-free medium or phosphate-buffered saline) and incubated for an additional 12–24 h in serum-free media. Conditioned medium containing secreted proteins is collected, centrifuged at 300 *g* to remove floating cells and gross cell debris, then further passed through a 0.22-μm filter to remove small debris. Conditioned medium is then concentrated up to 200-fold using a centrifugal filtration device (usually with a 3-kDa molecular weight cutoff). Protein concentration is measured and usually 50–100 μg of total protein is fractionated by SDS-PAGE. The resulting lane is sliced into 30–35 bands for in-gel digestion and LC-MS/MS analysis. Resulting data are searched against the desired protein database using a suitable search engine (such as Sequest or Mascot). The obtained list is then further curated for protein subcellular localization using the Uniprot knowledgebase [104]. Unclassified proteins are checked for potential secretion using the SignalP server [105]. Other proteins can be searched manually for subcellular localization in the literature or exosome databases.

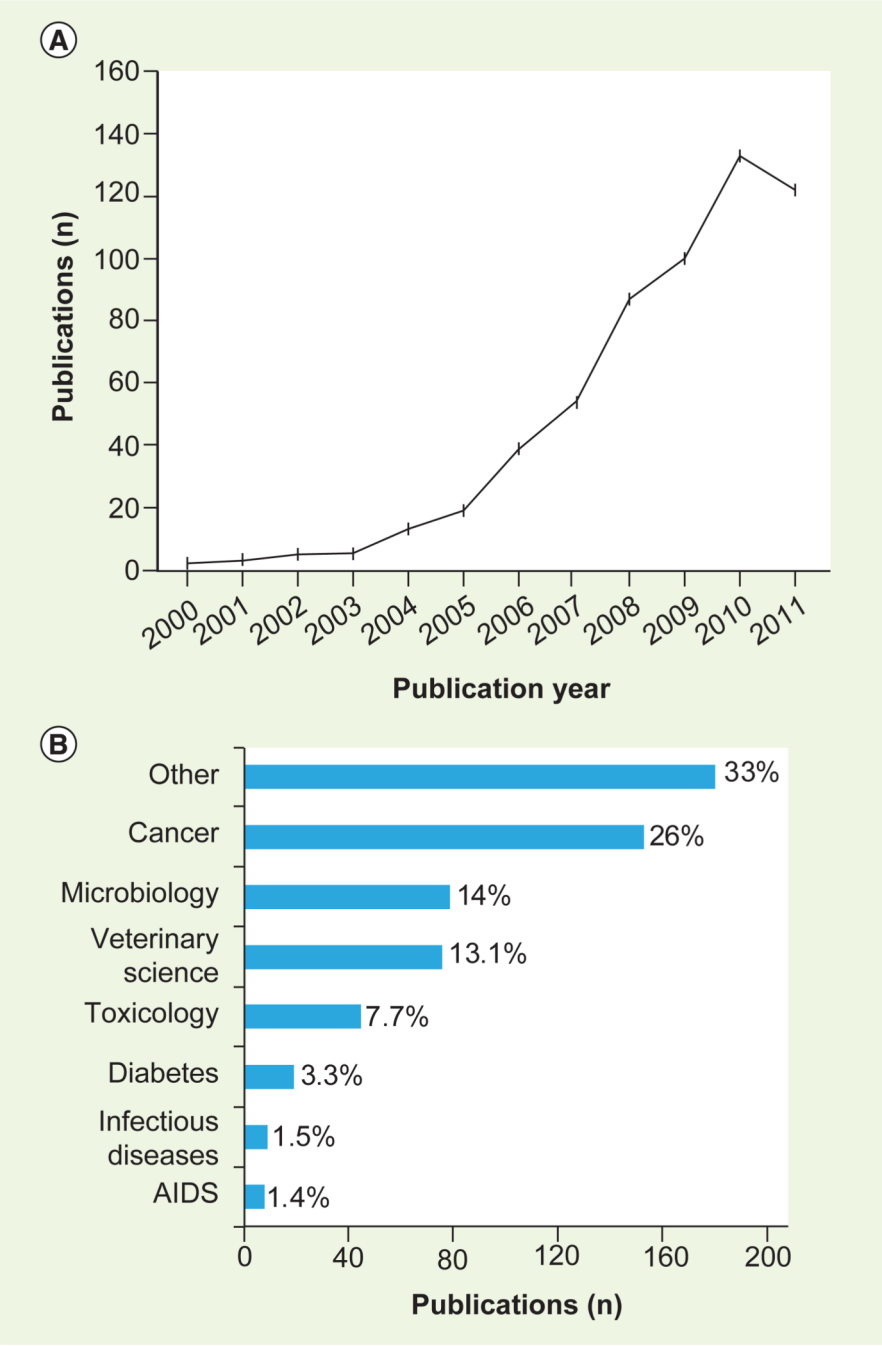

Figure 2. Numbers and disease distribution of secretome publications

(A) The number of articles describing secretome studies has been on the rise since the year 2000, when only one article was published, to the year 2011 when more than 570 articles were published. **(B)** A large number of secretome studies have dealt with cancer (26%) and microbiology (14%). Veterinary medicine, including murine models, constituted 13.1%, toxicology 7.7%, diabetes 3.3%, infectious diseases 1.5% and AIDS 1.4% of the published articles. Data were obtained by using the NCBI Pubmed Central database. Limits were set to human and animals and queried searching the word ‘secretome’ and manually verified for

inclusion. To generate disease-specific secretome studies, disease type (e.g., cancer) was included in the limits.

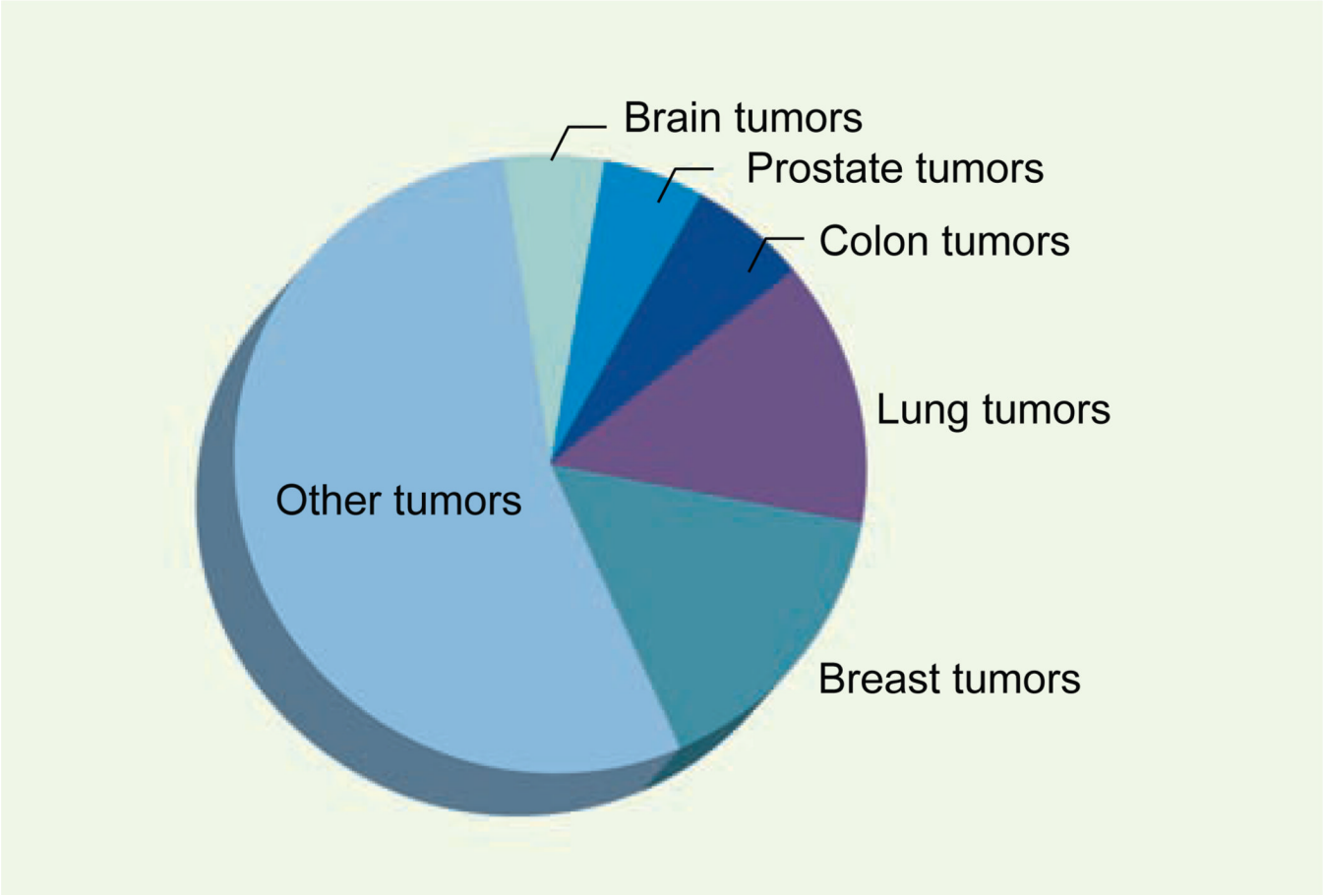

Figure 3. Pie chart distribution showing secretome profiling studies by cancer type
Data were obtained by using the NCBI Pubmed Central with a query of keywords 'secretome' and cancer type.

Table 1

List of some relevant secretomics studies and outcomes.

Study (year)	Field of study	Outcome	Ref.
An *et al.* (2010)	Biomedical science	Defined the role of a serine protease in age-related macular degeneration	[1]
Formolo *et al.* (2011)	Cancer	Defined potential proteins involved in brain tumor invasion	[2]
Korpal *et al.* (2011)	Cancer	Defined a novel mechanism of breast cancer metastasis	[3]
Wu *et al.* (2010)	Cancer	Defined a useful set of potential biomarkers for cancer in general and for specific cancers	[5]
Bobadilla Fazzini *et al.* (2011)	Metal extraction	Defined novel proteins to improve bioleaching and metal extraction	[8]
Bernay *et al.* (2009)	Brain	Discovered new bioactive neuropeptides	[11]
Jami *et al.* (2010)	Biotechnology	Improvement of penicillin production	[18]
Herpoel-Gimbert *et al.* (2008)	Biotechnology	Characterization of *Trichoderma*-secreted cellulases for the biofuel industry	[21]
Maxwell *et al.* (2008)	Cancer	Defined role of intracellular secreted proteins in tumor progression	[26]
Pant *et al.* (2012)	Biomedical science	Comprehensive review of the role of exosomes in health and disease	[29]
Gronborg *et al.* (2006)	Cancer	Defined potential pancreatic cancer biomarkers	[36]
Roelofsen *et al.* (2009)	Technique	Developed novel method to study tissue explant secretomes	[60]
Takeda *et al.* (2011)	Technique	Developed novel technique to study *in vivo* secretome in the brain	[70]
Lum *et al.* (2011)	Bioinformatics	Developed a comprehensive knowledge database for the fungal secretome	[72]