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Review

Tumor Interstitial Fluid: Proteomic Determination as a Possible Source of Biomarkers

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Abstract. *Tumor interstitial fluid (TIF) is formed largely by an imbalance between the forces that govern the filtration of liquid between the luminal and abluminal parts of tumor neovessels. TIF is a dynamic solution that varies according to tumor type, and is generally rich in proteins, lipids, and various enzyme-derived substances. These enzyme-derived substances can have important roles as both regulatory and inflammatory factors. Furthermore, the oncotic pressure caused by the presence of these proteins and peptides in TIF leads to a pro-inflammatory condition in which macrophages produce cytokines such as Interleukins 1 and 6. With the recent advent of proteomics, TIF has been studied extensively and can be used as a source of potential biomarkers for cancer, including breast, ovarian, and head and neck cancer. In the present review, we discuss the process of TIF formation, its composition, the effects of its accumulation, the methods of sampling, and the proteomic analyses performed on it, which make TIF a valuable tool in monitoring several cancer types.*

Gullino *et al*. defined the interstitial compartment of a tumor as all that is interposed between the plasma membrane of neoplastic cells and the vascular wall of the newly formed neovessels (1).

Studies on tumor interstitial fluid (TIF) were initiated in a scientific manner by Gullino's group in Bethesda, USA (2) and the Sylven group in Sweden (3). Although each group obtained TIF by different methodologies, Gullino's group (4, 5) scored and analyzed TIF with a process allowing for a state more closely resembling *in vivo* pathophysiology [*e.g*. Guyton *et al*.] (6). The method used by Gullino's group is best described as a pathophysiological method (4, 5), using a chamber enclosed within the tumor mass (described in further detail later). Ultimately, Gullino's process was able to determine quite accurately not only the pharmacokinetics of chemotherapeutics and monoclonal antibodies, but also variables such as interstitial pressure, TIF composition, tumor metabolism, and pH (bicarbonate). More recently Gromov *et al*. (7) and Wiig's group (8) have contributed additional methodologies to further obtain and study the composition of TIF.

More recent developments in proteomics have significantly changed the level of interest in the composition of TIF, potentially using it to diagnose and monitor neoplastic diseases through the identification of new tumor markers within TIF. In this review, we describe the various methodologies currently being used to obtain TIF, the current science in the proteomic analysis of TIF, and the potential for future applications.

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Pathophysiology of TIF Formation

The main function of the microcirculation is to transport nutrients and gases (*e.g*. O_2, CO_2), and to remove the waste products from tissues. This important function is carried-out in a specialized portion of the circulatory system, which includes a semi-permeable endothelium allowing for the exchange between the endothelial lumen and the interstitium. The movement of solutes between the luminal and abluminal regions of the endothelium (9), and its subsequent reabsorption was first formulated by Starling in 1896 (10, 11). The Starling equation states that the distribution of solutes in the microcirculatory unit results from the differences in capillary and interstitial hydrostatic pressure, plasma and interstitial colloid osmotic pressure, and the vascular wall permeability [Equation (1)]:

$$Jv=(Lp\ S)[(Pc-Pi) - \sigma\ (\pi c-\pi i)] \qquad \text{(Eq. 1)}$$

where Jv is volume flux of fluid (ml/min); Lp is hydraulic conductivity ($cm \times min^{-1} \times mmHg^{-1}$); S is the capillary surface area (cm^2); Pc and Pi are capillary and interstitial fluid hydrostatic pressures, respectively (mmHg); πc and πi are capillary and interstitial colloid (oncotic) pressures, respectively (mmHg); and σ is the osmotic reflection coefficient of the vessel wall (σ is 0 if the membrane is fully permeable to molecular species and 1 if the membrane is impermeable) (11). According to Starling, these forces are constantly modified according to Equation 1. For example, there is a dynamic variation in osmotic pressure across the endothelial axis, particularly between the arteriolar and venous end. Another example of the factors affecting Starling's law is the changes which can occur in endothelial permeability, particularly in inflammatory and tumor disease states (12, 13). Endothelial permeability also depends on the levels of endothelial glycocalyx, which is a network of membrane-bound proteoglycans and glycoproteins which cover the luminal endothelium (14-17). Taking these two variables into consideration, the revised Starling equation is as follows (14, 15):

$$Jv=(Lp\ S)[(Pc-Pi) - \sigma\ (\pi c-\pi g)] \quad \text{(Eq. 2)}$$

where πi is replaced with πg, or the difference in oncotic pressure of the thin layer of interstitial fluid between endothelial cells immediately beneath the glycocalyx covering the endothelial surface (14, 15). The difference in oncotic pressure is not merely the difference across the endothelium and interstitium, but across the glycocalyx (14, 15).

In the tumor microenvironment, the anticipated increase in TIF has been demonstrated in both animal and clinical studies (4, 5, 12, 18). This increase in TIF results from the failure of the aforementioned physiological laws described by Starling on the regulation and exchange of fluids within the setting of the tumor microcirculation. There are several reasons for this failure, which we describe in detail, including: (a) tumor capillary structure and physiology; (b) similitudes with inflammation and edema formation in other disease states; (c) lack of lymphatic drainage.

(a) Differences in structure and physiology of tumoral and normal capillaries. Several structural differences exist between tumor and normal capillary endothelium. The first is that tumoral endothelial cells forming neo-angiogenic vessels do not form a normal monolayer, but instead, are irregularly shaped, disorganized and even overlapping one another (19). In addition, there are loose interconnections between these cells, with focal intercellular openings of 2 μm in diameter under electron microscopy (20).

The second difference can be found in the pericytes which exhibit irregular behavior in the regulation of vascular formation, stabilization, remodeling and function of tumoral vessels (21, 22). These dysfunctional pericytes deposit extracellular matrix components into the perivascular region, ultimately generating a defective basement membrane (22, 23). Furthermore, they participate in the expression of various cytoskeleton proteins which extend their cytoplasmatic processes deep into the tumoral tissue (23). Pericytes therefore also contribute to the loosening of interconnections between endothelial cells, permitting further neo-vessel growth and metastasis (21-25).

The third difference is that the tumor itself produces not only pro-angiogenic factors such as vascular endothelial growth factor (VEGF), but also vasoactive factors such as bradykinin, nitric oxide and its derivatives the peroxynitrites (26, 27). These vasoactive factors make the tumoral endothelial cells even more dilated, permeable and leaky (19, 28, 29).

Lastly, tumoral neo-vessels are tortuous and lack the normal hierarchical arrangement of arterioles, capillaries and venules (29). This renders them more leaky and disorganized, further enhancing the efficiency of fluid exchange between the vascular and the interstitial space (30). This imbalance increases the tumor interstitial space, and ultimately the volume of TIF, as demonstrated in several animal and human tumor types by Gullino (5) and Jain (31).

Other morpho-functional abnormalities, such as a higher number of arteriovenous anastomoses, an increased tortuosity of tumor neovasculature and stagnation of blood flow (31, 32) determine alterations of pressure along the capillary length. The main force regulating fluid filtration is the capillary pressure Pc, which is normally between 28 and 35 mmHg, with an axial decrease along the length of the vessel of 15-20 mmHg. This drop is called the arterial venous pressure difference (a-v Δp). This difference is lesser within tumor capillaries than in normal capillaries, with Pc at the arterial end approaching that of the venous end, with a median drop of 8-10 mmHg (30, 31). This develops a gradient at the venous end for πi compared to Pi. This behavior is not completely understood but can be attributed to factors including decreased re-absorption together with tumor vessel leakiness, causing a flow of macromolecules toward the interstitium, as opposed to normal steady-state physiological conditions (18, 33). Albumin generates 70% of the oncotic pressure, but is lost in a greater quantity, leading to an increased oncotic pressure (πi)(osmolarity) in the interstitium, as demonstrated in animal studies (4, 31, 34). Levick and Michel have shown that decreased water re-absorption is not only influenced by osmotic pressure, but also the reflection coefficient for albumin (which is less than 1) (11). Of note, osmometers yield falsely elevated osmotic pressures compared to *in vivo* osmotic pressures, presumably due to their static membranes. Of course, microvascular absorption is transient and filtration occurs even in steady states, since amongst others, pores increase in number and size. This is especially true in inflammatory states such as

cancer, where there is typically increased fluid filtration (11). These additional factors lead to abnormal lymphatic drainage, and further accumulation of TIF, increasing TIF pressure above and beyond osmotic pressure (12, 13, 35).

(b) Similitudes of TIF formation with inflammation and edema formation in other disease states. Several authors have questioned Starling's law and its validity, overall, in various disease states such as edema and inflammation. These authors have given much importance to the interstitial pressure present in these states and the behavior of the extracellular matrix, also stressing the increased endothelial permeability. These authors showed (12, 13) that the formation of interstitial fluid during an inflammatory state is sudden, and that filtration coefficient augments hundreds of times. The increased permeability, particularly evident in tumors, is due to the inflammatory reaction always present and to the increased production of VEGF. Initially, interstitial pressure decreases and promotes flow towards the interstitium, then the interstitial pressure increases and limits the formation of edema. The interstitial inflammation in neoplasia does not behave in a passive way but participates in the formation of edema and then of interstitial fluid. In fact, the composition of the interstitial fluid (proteoglycans, collagen fibers and hyaluronic acid) causes it to act like a sponge that holds and sucks water from the capillary bed. This creates a vicious cycle similar to that of the self-sustaining hypoxic state. The latter is then responsible for the increased production of VEGF and recruitment of inflammatory cells (myeloid-derived suppressor cells, and macrophages) and the production of inflammatory cytokines such as Interleukin-1[IL-1], Tumor necrosis factor [TNF] and Prostaglandins E2, which contribute further to create differences in hydrostatic and osmotic pressures between tumor and the interstitium (12, 13).

(c) Lack of lymphatic drainage. Lymphatics are present at the tumor periphery and are largely not found within the tumoral mass (4). In agreement with Kesler *et al*. and others, we have found their structure to be akin to lymphatics inside tumoral masses, but these apparent lymphatics are compressed and ultimately are nonfunctional (36, 37). This does not permit fluid reabsorption in tumoral neo-vessels, and along with the above factors, this progressively increases TIF pressure from the periphery to the center of the tumor. This increase is the propelling force that pushes lymphatic fluid through the lymphatic vessels, favoring metastasization and immune suppression (4, 11, 38-40). Other factors also contribute to these processes and are reviewed by Kesler *et al*. (36). These factors are principally elicited by the hypoxic environment of the tumor, due to the increased production of nitric oxide, and the chronic anatomical enlargement associated with inflammatory conditions and immmunosuppression (see Figure 1) (36, 40, 41).

Methods for Obtaining TIF

To study the composition and the variability of TIF quantitatively and qualitatively, several methods have been developed. In order to describe the methods of studying TIF from the point of view of normal physiology, we use the works of Guyton *et al*. (6), Gromov *et al*. (7), Wiig and Swartz (12), and Haslene-Hox *et al*. (38). The methods can be divided and compared according to how each of these experts isolated TIF *in vivo* [capsule implantation, and catheter insertion (wick-in-needle technique, microdialysis, glass capillary, capillary ultrafiltration)] and their methods for obtaining TIF from fresh tissue specimens [tissue centrifugation, tissue elution, Capillary. liquid chromatography-tandem mass spectrometry (LC-MS/MS)].

a) In vivo methods. Capsule method: Until 1960, the pressure of the interstitial fluid was always thought to be positive, when Guyton *et al*., with the advent of the implanted capsule method, proved that the pressure of the interstitial fluid was in fact negative (6). Before 1960 two methods were used: intra-tissue balloon technique and the needle or capillary pipette technique. Guyton *et al*. were critical on these two methods. The balloon method measured the total pressure (tissue plus TIF pressure) and not just the pressure of the interstitial fluid. In the micro-capillary or needle method, the size of the needle or the capillary is far greater than the space occupied by the actual interstitial fluid, and does not account for the distortion of space and measurements *in vivo*. Guyton *et al*. developed new techniques for measuring interstitial fluid pressure between 1960-1967, including the capsule method. This method allows for a large area of exposure to interstitial fluid, allowing an equilibrium pressure to be reached which is not collapsible, creating a possibility for obtaining a specific amount of the liquid for analysis. Initially, there is extravasation of blood as a result of the installation of the perforated capsule, followed by an inflammatory reaction for a period of 2-3 hours. With the reduction of inflammation, the pressure becomes negative, and can be measured by means of a needle introduced into one of the perforations (6). Building on these improved methods of Guyton *et al*., Gullino began to create the capsules that could both allow for the measurement of the pressure and for obtaining a certain amount of tumor extracellular fluid. The main intent of Gullino was to create a tumor model that behaves like an organ connected to the host by a single artery and vein (Figure 2) (5, 6) Gullino *et al*. planted tumor cells on rat ovary so as to obtain a tumor mass that could be studied as an isolated organ (5, 6). The technique is not easy and requires a highly skilled staff, but obtaining an organ-isolated tumor can provide a wealth of information. With the growth of the tumor on the ovary as the first step, the second step is to insert a capsule similar to that of Guyton *et al*., in order to obtain the interstitial fluid and pressure. The

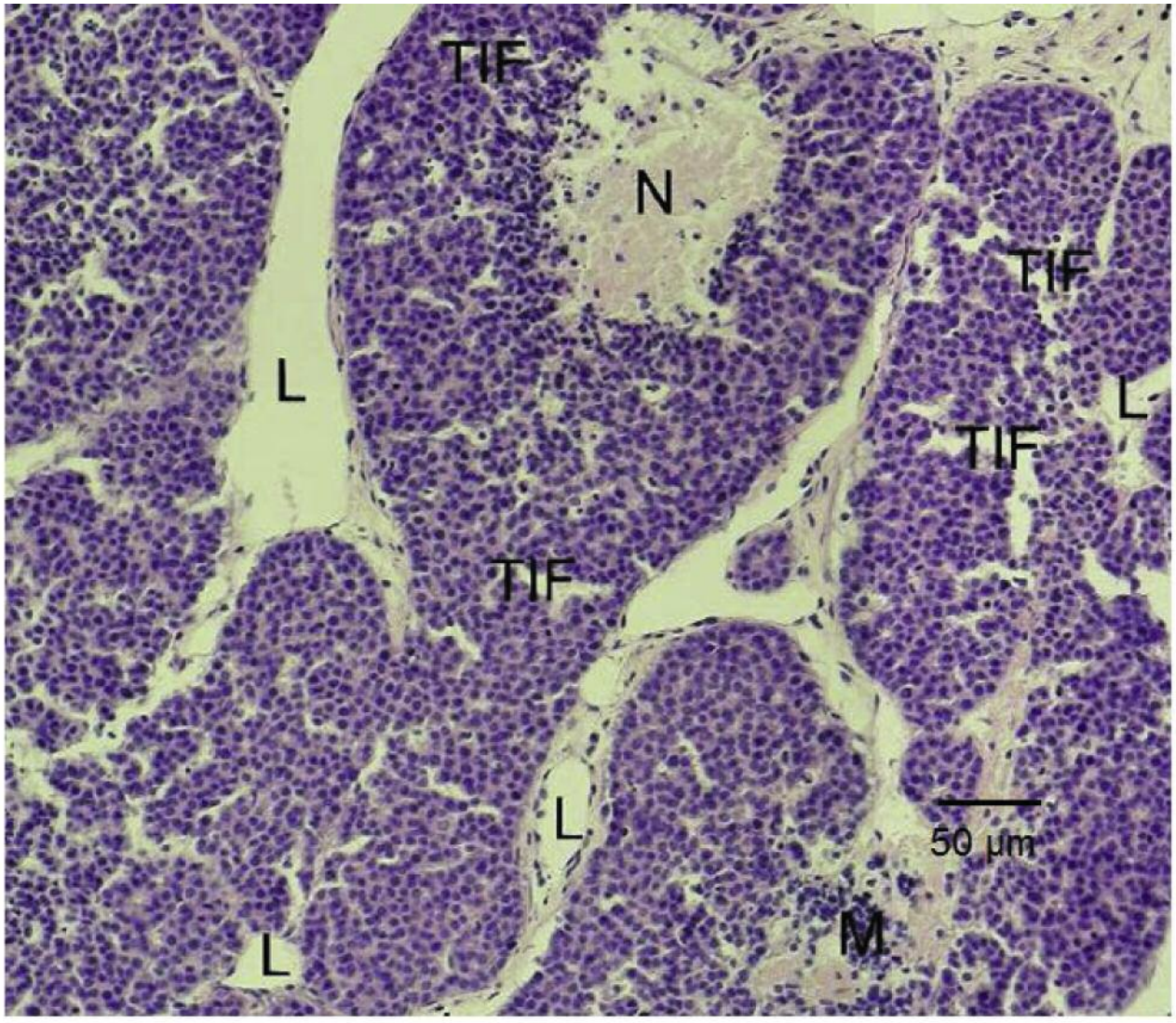

Figure 1. *Chronic lymphatic enlargement (L), associated with a pouch of Tumor interstitial fluid (TIF) and macrophages are illustrated around the neoplastic tissue (N). Macrophages represent the inflammatory infiltrate. (Mammary tumor developed in a MMTV-neu (erb-2) transgenic mouse) (Figure kindly supplied by Isabel Freitas, University of Pavia, Italy).*

capsule is constituted by a ring of 1-2 mm (a tiny cylinder of plexiglas) at the two ends (upper and lower) of which are glued two millipore membranes; this tiny cylinder creates a small cavity connected to the outside to obtain pressure measurements and fluid sampling through a tiny catheter (see Figure 2). The space differs slightly from that of Guyton *et al.* due to the fact that the chamber is inserted into the growing tumoral tissue and is left until it becomes one with the tumoral mass. This allows interstitial fluid to be obtained throughout the tumoral evolution, verifying its pressure, its composition and its variations by changing the biochemical parameters such as pH and glucose.

Microdialysis method: This is a method invented principally for obtaining and measuring constituents present in extracellular fluids (42). This methodology involves the insertion of a microcatheter (probe) in the tissue being sampled. This probe is formed by two concentric tubes, within which is a liquid continuously perfused at low flow rate (0.5-10 $\mu l \times min^{-1}$) (43).

A semi-permeable membrane is present at the tip of the probe. The molecular weight cutoff of these membranes is approximately 20-100 kDa (44). Membranes with a cut-off of 20 kDa are used for sampling small metabolites such as glucose or glycerol, whereas a cutoff of 100 kDa is used for

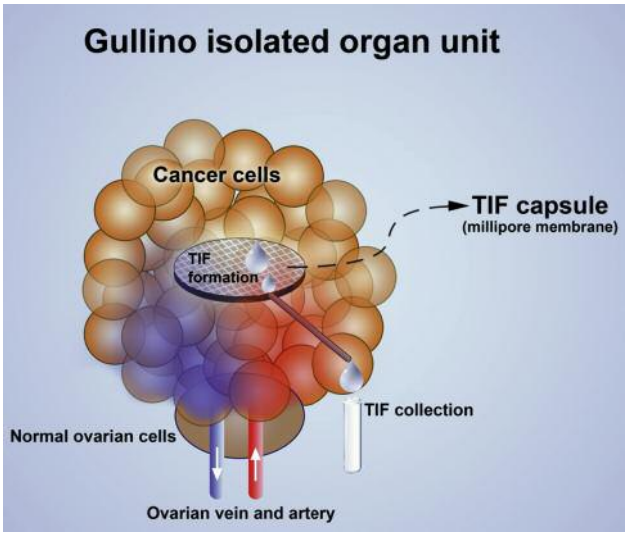

Figure 2. *The isolated organ unit of Gullino et al. is illustrated. Cancer cells initially grow over normal ovarian cells then they envelop it as the capsule that become a newly formed tumoral structure. As illustrated by arrows, there is an entrance ↑ (ovarian artery), an exit (ovarian vein) and a collector of Tumor Interstititial Fluid (TIF).*

macromolecules (45). The substances present in the extracellular fluid diffuse into the liquid perfused through the semi-permeable membrane and are collected in the dialysate. The chemical composition and concentration of the substances present in the TIF are analyzed, and the concentration in the extracellular fluid is calculated (43-46). Special precautions must be taken for sampling macromolecules and cytokines (47,48). The technique has been used for studying metabolites and drugs in the brain, in lung, skin, and adipose tissue (43-46). The technique has been used both in physiological and pathological states, such as tumors (43, 44). These authors have utilized this technique for studying proteins, peptides and cytokines secreted by the tumor and those factors governing tumor communication in the tumoral microenvironment (44). Other authors have studied an on-line procedure to ameliorate issues with data acquisition (49). The on-line procedure is particularly important for studying the substances produced by the tumor (secretomes) through proteomics. There is a sequence from the acquisition of the dialysate through the microdialysis technique to the separation of products by liquid chromatography (LC) and finally their analysis by mass spectrometry (MS) (49). Many substances from glucose to drugs and neurochemicals can be studied, and various procedures have been adapted for following the substances to be studied. The authors discuss the advantages and the disadvantages of this on-line technique (49). Haslene-Hox *et al*. (Wiig's group) (8) consider microdialysis a good technique, as it allows studying for both *in vivo* tumoral tissue (especially the brain) and drug distribution in a continuous and repeatable way. However, all methods for microdialysis have two disadvantages; firstly that at the point of insertion of the catheter, an inflammatory reaction will be produced, disturbing fluid collection; and the other is that the recovery of macromolecules in the dialysate is rare.

Wick method: This technique has been utilized by Aukland and Fadness (50) and applied to measure osmotic pressure and pressure in tumoral tissue (34). The wick-in-needle technique consists of a hypodermic needle inserted into the tumoral mass, and filling the interior with nylon sutures soaked with a saline-buffered solution. The wick needle can be placed acutely in cases of pressure measurement, with a pressure transducer being used at its end (34, 51). For obtaining TIF, the needle is placed in the same way as for measuring intra-tumoral pressure, in an acute or chronic way, but the pressure transducer is substituted by a sample collector. This method, is not free of disadvantages such as bleeding, cellular damage, and inflammatory reaction (7).

Glass capillary: The method was used for the first time by Sylven and Bois (3) for obtaining interstitial sample in a simpler way compared to Gullino's method (4, 5). As reported by Wiig (52), the method was used because Sylven and Bois (3) noted that the tumor periphery was rich in edema-like interstitial fluid, which was easily obtainable by glass capillary insertion. Simplicity however does not always translate into accuracy. In fact, the insertion of a capillary tube can also collect tissue and fluid from the tumor margins from normal tissue and vascular tissue, thus the liquid collected may be not true interstitial fluid.

Capillary ultrafiltration: This technique and various applications have been described by Leegsma-Vogt *et al*. (53). Ultrafiltration avoids complicated and time-consuming recovery calculations that are necessary when using *in vivo* microdialysis (53, 54). The force which allows for the collection of the interstitial fluid is a negative pressure capable of extracting the liquid. This negative pressure and the permeability of the membrane are, however, limitations of the technique (7).

b) Methods for obtaining TIF from fresh tissue specimens. Tissue centrifugation: Since 1989 Wiig *et al*. have been trying to obtain interstitial fluid from fresh tissue in order to study its composition, pressure and the osmotic force exerted, starting with corneal tissue (52, 55). Utilizing the same technique, they were able to isolate interstitial fluid from a rat mammary tumor (55). The TIF is isolated using centrifugation apparatus exposing the tissue to an increase in G-force (8). The main question asked by Wiig's group was whether during centrifugation the liquid obtained was representative of the TIF. Using appropriate tracers, they concluded that the liquid obtained with their methodology

was indeed representative of the TIF (8). After this validation, the technique was used by Haslene-Hox *et al*. for other tumors, such as metastatic ovarian tumors, inflammatory tumors and ovarian tumors (8). The method has proven highly suitable for proteomics analysis.

Tissue elution: This methodology was established by Gromov's group for obtaining passive extraction of interstitial fluid from a surgical small preparation (7). The procedure consists of obtaining, small pieces of tissue (1-3 mm^3) from a small tissue biopsy (0.25 g) which are then washed in a phosphate buffered saline solution (PBS), and to put them in a plastic container with PBS. These samples are kept for 0 to 24 h in a CO_2 incubator at 37˚C. The tissues are then centrifuged at 4˚C at 2800 × *g* for 20 min and the supernatants obtained are kept at −80˚C for further examination. Different buffer solutions such as a solution of Dulbecco's or Eagle's medium have also been studied, but no significant differences have been found (7). The only apparent disadvantage of the technique thus far is the possible contamination of intracellular components caused by cutting the tumor tissue. The method has been used for several tumor types and is an integral part of the approach to proteomic analysis of TIF used today by Gromov *et al*. (7). See Wiig and Swartz for a complete list and comparison of various techniques reported here (12).

Capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). Another technique for collecting TIF *in situ* was used by Stone *et al*. (56). These authors used a hollow fiber catheter developed by Ohlfest's group for improving drug distribution to the brain (57). The catheter permits the passage of fluids but prevents the passage of solid material and it is connected to an infusion pump connected to a vacuum, allowing for extraction of fluid (56). The TIF obtained was used to generate the first catalog of proteins from a head and neck squamous cell carcinoma (56). To maximize proteomic coverage, TIF was immunodepleted for high-abundance proteins and digested with trypsin, and peptides were fractionated in three dimensions prior to MS.

TIF Composition and Source of Proteins, Exosomes, Growth Factors and Secretomes

Gullino's group in Bethesda was the first to systematically analyze TIF in transplanted animals for composition and its role as a modulator of cancer progression. The aforementioned method used by Gullino of implanting a capsule for thee isolated tumor perfusion models (1, 2, 5, 58), was used principally in four types of transplanted tumors in rats: hepatomas, Walker carcinoma, fibrosarcoma, and Novikoff hepatoma. The interstitial water space of all specimens studied was found to be very large, between 32 and 60% of the tumoral water, depending on the tumor type. In hepatomas, the interstitial water space represented 40 to 55% of the tumoral water and was three to four times larger than that of the liver (2, 4).

TIF differs with respect to aortic plasma in terms of glucose, lactate, cholesterol and lipid phosphorus content, the concentration of protein is lower (peculiarly fibrinogen was found to be lacking and never coagulated) and that of free amino acids was higher in the TIF than in aortic plasma (5, 18). Alpha-globulins were reduced compared to serum but the albumin:globulin ratio of the TIF was equivalent to that of serum (1, 18). The higher level of hyaluronidase activity in TIF compared to subcutaneous areas distant from the tumor was presumed to contribute to the low concentration of hyaluronic acid in TIF. The pH of TIF was 0.2 to 0.4 units lower, the pCO_2 16 to 39 mmHg higher, dissolved CO_2 about 1 mM higher, and bicarbonate concentration 4 to 6 mM higher, compared to plasma of the blood afferent to the tumor (1, 2, 4, 5, 59). Other factors studied by Gullino's group were collagen content, gangliosides, copper and prostaglandins (especially of E_1 type) (60). These authors noted that the concentration of certain gangliosides GM1, GD1b and GT1b, had antiangiogenic activity and that prostaglandin E1 type plus copper were essential in the process of angiogenesis (61-63). Albumin was demonstrated by them and other authors to be lost abundantly, due to increased permeability, similar to the outflow of other macromolecules, such as matrikines, exosomes and other microparticles (5, 18, 64). In a certain sense TIF must be considered a 'container', where associated with the wastes of cells you can find useful molecules that may become a distinct source of information. Matrikines are peptides liberated by partial proteolysis of extracellular matrix macromolecules and which are able to regulate cell activities (18, 64). Exosomes are special carriers/vesicles ranging in diameter between 50-100 nm, floating in a sucrose gradient between 1.13 and 1.19 g/ml (65). Exosomes are pieces of biological membranes released by many type of cells and are the end product of biochemical processes, which means they can contain many proteins, lipids and genetic materials (65-67). They are nanoscale messengers containing a variety of different molecules from signal peptides to microRNA, and lipids. They are either released into the extracellular fluid or may enter circulation, resulting in an increase in numbers of regulatory T-cells (67, 68), tumor progression (69), and tumor immune evasion (70). Exosomes have other important functions beyond immunity, angiogenesis, and thrombosis (71). They can also transfer much information that facilitates the survival and the expansion of regulatory T-cells (67).

Secreted proteins, as reviewed by Harthout, are a class of proteins that have an important regulatory role in many biological processes (72), and that if studied appropriately, may become a significant source for useful biomarkers in oncology. Park *et al*. (73) reported that mammary tumors

secrete specific proteins (predominantly cytoplasmatic and membranous proteins) and exosomes that may facilitate angiogenesis and metastasis (73).

Increased extracellular osmolarity, of TIF is linked to the abundance of albumin and other polypeptides lost into the TIF due to increased tumoral vascular permeability. As described by Brocker *et al*., increased osmolarity acts as a potent inflammatory stimulus (74). Many cells have developed methods of adaptation to changes in osmolarity, such as cells of the kidney, but others, such as macrophages, respond to hyperosmotic stress with an increased production of proinflammatory cytokines (Interleukins 1, 6 and 8, and Tumor necrosis factor (75). Nèmeth *et al*. pointed out that increased osmosis stimulates the activation of nuclear factor kappa-B in intestinal epithelial cells (76). On the other hand, Gupta *et al*. demonstrated the increased activation of nuclear factor kappa-B as being almost always a biomarker of chronic inflammation and playing a critical role in tumor growth (77).

Chenau *et al*. reviewed the three different mechanisms by which proteins are secreted by cells (78). In the first mechanism, the secreted proteins are conjugated with a signal peptide of 15-30 amino acids and then carried through the endoplasmatic reticulum and transported to the Golgi apparatus to the plasmatic membranes, at which point they are released by exocytosis. In the second mechanism, proteins are secreted without the signal peptide through pre-existing endosomal vesicles followed by classic exocytosis or translocation from the inner to the external part of the membrane. In the third mechanism, proteins are released inside exosomes (78).

Proteomics

According to Cho, the study of secreted proteins is more complicated than the study of DNA and RNA (79). This is due to different biochemical characteristics of the proteins, such as their secondary and tertiary structure, their relatively easy denaturation by enzymes and their solubility (79). To analyze these secreted proteins or other proteins of the TIF requires two steps: firstly a step of separation, as we described in the section on methods to obtain TIF, and secondly of identification (78). Identification of secreted proteins has been greatly facilitated by the advent of proteomics (80, 81). However the analysis of TIF by proteomics is complex and there remain many limitations in its science and technology. As outlined by Gromov *et al*., there is a need for the creation of standard methods of tissue dissection, sampling and detection (7). The complexity of TIF study by proteomics derives from several factors linked to difficulties in studying the pathological state in vitro, and with the known limitations *in vivo*. A current list of factors known to contribute to the determination of this complex science of proteomics is illustrated in Table I (7).

Table I. *List of factors that may limit determination of the proteomic profile of tumor interstitial fluid (TIF) and the search for biomarkers [adapted from Gromov (7)].*

TIF sampling
Abundance of proteins
Multiple Interactions and secretions of proteins by different cells at the same time
Presence of same proteins in different body fluids
Folded and unfolded proteins
Proteomic methods of analysis

As reviewed by Gromov *et al*. (7), several principal proteomics methodologies have been used to characterize TIF. They are: gel-based proteomics/non gel-based proteomics, (chromatography), array-based proteomics, peptide-centric proteomics, and MS.

As outlined by van Riper *et al*. (82), for characterizing proteins it is necessary to program an analytical platform able to quantify protein abundance, post-translation modifications and to reveal the members of protein complexes. MS, coupled with other technologies allowing for sample fractionation and automatic methods of analysis, provides such a versatile and powerful platform (82).

The first step in the study of proteins is their separation and isolation (83). Several procedures exist for separating protein mixtures but the one most commonly used is polyacrylamide gel electrophoresis (PAGE) (83, 84). The aim of PAGE is to separating complex mixture of proteins according to their electrophoretic mobility. The mixture of proteins is generally induced to migrate in a reproducible way by applying an electric field, and is then immersed in a denaturant, generally sodium dodecyl sulfate (SDS). An improvement of this technique is 2D-PAGE, which takes into consideration not only electric mobility but also molecular weight. 2D-PAGE has permitted the identification of new proteins, and comparison of their concentration between samples. It is an economical method, with good resolution, however, some limitations do still exist. 2D-PAGE cannot quantify small amounts of proteins, or proteins too small, too large, too basic or too acidic (84-87). Once the proteins are separated, they must be quantified through different staining techniques. The development of fluorescent dyes has improved the sensitivity of protein detection as the pH gradient is changed (85, 88).

Another technique was developed more recently called 2D fluorescence-difference gel electrophoresis (2DIGE) saturation labeling technique coupled with quantitative image analysis software DeCyder™ (GE health care Life Sciences). This tecnique is able to reveal numerous samples concurrently in the same 2D process, minimize gel to gel variation, and to compare the protein features across different gels by means an internal fluorescent standard. This

innovative technology relies on a pre-labeling of protein samples before electrophoresis with fluorescent dyes. These dyes exhibits a typical wavelength fluorescent that allows more experimental samples to be studied. Thus, the samples can be separated simultaneously in a single gel (89). The internal standard, which is a pool of an equal amount of experimental protein sample, can facilitate data accuracy in normalization and increase the statistical confidence in determining the relative quantity across gels (89). Further efforts have afforded automation of this process (90), improving speed and resolution (79).

Mass spectroscopy: Proteomics-pattern profiling is a recent approach in the discovery of biomarkers and in use for a personalized therapeutic approach. Compared to genomics, proteomics looks more promising in the study of biomarkers. This is related to the fact that proteins secreted by tumor cells or near its microenvironment are poured into the circulation and can be measured by MS. The methods of proteins analysis by mass spectrometry have improved and include advanced technologies that require minimum sampling and allow different proteins to be analyzed in a short time. The new forms of proteomics have a greater sensitivity and specificity (87, 91-94).

For developing new diagnostic tools and improving patient care, a tandem LC-MS/MS method has been used to map the human proteome. The principal MS techniques are matrix-assisted laser desorption/ionisation (MALDI)–MS or the closely related surface-enhanced laser desorption/ionization (SELDI)–MS, can allow the surface upon which ionization takes place to provide a degree of fractionation owing to variable absorbance of peptides (91-94).

These high-throughput techniques have been widely deployed for cancer biomarker discovery, especially for the measurement of low-Mw protein/peptide fragments (Mr <20kDa) in blood. These proteolytic fragments, including those derived from abundant plasma proteins, are often referred to as the blood peptidome. Non-identity-based SELDI–MS involves comparative profiling (pattern-matching) of MS-derived peptide ion patterns purportedly derived from *in vivo* proteolytic cleavage of plasma proteins, low-abundance tumor-derived proteins or products of tumor-derived proteases seized by albumin (91-95).

These blind-screening methods involve a comparative analysis of polypeptide peaks of different mass/charge ratios that differ in intensity between the blood of patients with cancer and those of healthy individuals. Biomarker discovery studies of this nature have drawn cautionary notes owing to the problems in experimental design and data analysis, or in biases related to blood collection, processing, and storage protocols (94).

Without identification of the peptides/proteins accounting for the MS-derived ion patterns using SELDI– time-of-flight–MS, it is very difficult to interpret the findings. To solve this problem, Villanueva *et al*. proposed that tumor-derived proteases initiate cleavage of plasma proteins that can continue *ex vivo*, and this phenomenon, combined with the physiological action of coagulation enzymes, produces serum peptidome patterns diagnostic for specific malignancies (95).

Protein microarrays: The techniques described above give information on quantity, but lack information on biological functions of proteins and on their involvement in the altered signaling pathways present in cancer cells (87). Microarrays can analyze different biological samples simultaneously, but lack international standardization, thus all data obtained through this technology must be validated through the use of more consolidated standard techniques such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), western blotting (87, 96). A protein microarray consists of a solid support (slide) on which different components (purified proteins, peptides, antibodies, allergens, *etc*.) are deposited in an orderly manner and to a specific and defined density (up to 500 molecules/spot of 150 mm). Each of these components capture their protein target, thus isolating it from a complex mixture. Captured proteins are subsequently highlighted and quantified or assessed regarding their activities (87, 97). Microarrays can be based on the use of antibodies (antibody array) or on the use of antigens or autoantigens (98, 99). Once proteins are captured by microarray technique, they must analyzed and detected. As outlined by Berrade *et al*. (97), detection methods must have some important characteristics for providing specific reliability, high signal-to-noise ratio, good resolution, high dynamic range and reproducible results, and relatively low costs of management (97). The methods generally used are the fluorescence-based or label-dependent, and the label-free. In the fluorescence-based method, protein detection is achieved by the so-called sandwich fluorescence-based detection system in which captured proteins are detected by a secondary fluorescent-labeled antibody (97). The method is simple and proven useful especially in the study of DNA (100). The fluorescence-based method, although simple and inexpensive, suffers from some limitations. They are: a) decreased binding affinity between reagents and proteins that must be captured, b) the technique is tedious and time consuming, c) decreased labeling efficiency. These factors can be overcome using a label-free method: MS (97).

Cancer Biomarkers Obtained by Analysis of TIF

A biomarker is a measurable biological indicator that can indicate the presence of a disease state. As reported by Mishra and Verma, cancer biomarkers can be either a molecule secreted by the tumor or a tailored response of the organism to the presence of cancer (101). Another aspect on the usefulness

of biomarkers outlined by Teng *et al*. is that they must have a concentration sufficient to be analyzed (102). Adequate concentrations can typically be found in fluid near the tumor, where they are secreted, in the extracellular fluid. Extracellular fluid according to Teng *et al*. (102) has some advantages because it can be collected or harvested from the same individual and consequently is easily comparable. In fact, the timber of proteins within plasma is known to exceed 1010, with many potential biomarkers present at lower than required protein numbers as reported by Jacobs' group (103). Nonetheless, TIF has not been widely chosen for the analysis of biomarkers, probably due to difficulty in obtaining a good sample. As reported by Haslene-Hox *et al*., the most widely used technique for obtaining TIF was the method of elution compared to that with centrifugation capillary filtration and microdialysis (8). The quantity of proteins obtained by Haslene-Hox *et al*. with their technique were six-fold higher than in patient plasma and suitable for obtaining biomarkers of ovarian cancer (8). The protein chosen as a biomarker was stress-induced protein 1 (STIP1). This stress protein was validated by Wang *et al*., who concluded that STIP1 combined with Cancer antigen 125 (Ca125) was able to increase the early detection of ovarian cancer (104). The TIF of 23 patients with a confirmed diagnosis of serous epithelial carcinoma was compared to those with normal interstitial fluid of 19 persons with normal ovaries. STIP1 is a secreted protein able to organize other chaperones and which promotes proliferation of ovarian cancer in particular (106). It has the capacity to activate the transcription of the inhibitors of DNA binding (ID) 1, 3 and 4 and to induce the phosphorylation of intracellular proteins SMAD1/SMAD5 that regulate transforming growth factor-β (105, 106). Another study by Stone *et al*. analyzed TIF from patients with head and neck squamous carcinoma (56). The TIF was collected by ultrafiltration and compared to other bodily fluids, peculiarly with saliva from healthy volunteers and databases on plasma. A total of 325 proteins with the PAGE method, western blot and ELISA were found to be candidate biomarkers. Among them, profilin seems a good biomarker of tumor progression. In fact, profilin has a specific role in cell migration (107). Gromov *et al*. have published important studies on breast carcinoma and bladder carcinoma (108-110). Both tumours have been recognized as good sources of secreted proteins in TIF. In breast carcinoma, the TIF was obtained by elution and compared with normal interstitial fluid. Out of the 630 proteins detected as abnormal with 2D PAGE (110), were up-regulated in breast cancer TIF compared to normal interstitial fluid. The up-regulated proteins found by Gromov's group were calreticulin, lactate dehydrogenase, glutathione-S-transferrase-π, thioredoxin, oxidoreductase-protein disulfide isomerase, triose-phosphate isomerase, elongation Factor 1- β (EF 1-β) and galectin 1 (7).

Calreticulin is a calcium-binding chaperone with multiple functions in the immune system as antigen presentation (111). Galectin-1 is a glycoprotein with a wide range of immune-regulatory activity (112).

Thioredoxin has a function in maintaining the redox state (113). Glutathione-S-transferase-has a role in intrinsic and extrinsic cancer drug resistance (114).

Oxidoreductase-protein disulfide isomerase acts as chaperone (115). Triose-phosphate isomerase has a role in glycolysis, particularly in breast tumors positive for human epidermal growth factor receptor 2 (HER2/neu) (116).

Sun *et al*. utilized elution to obtain the interstitial fluid of hepatocellular carcinoma and compared its composition with plasma from the same patients (117). They analyzed the secreted proteins by linear ion trap MS after SDS-PAGE separation and identified 1,450 proteins. Out of these proteins, 111 were specific for hepatocellular carcinoma, and 27% were up-regulated nuclear proteins (117).

Microdialysis for obtaining TIF or extracellular fluid is normally used for brain, Hardt *et al*. used this technique for head and neck carcinoma (118). They studied eight patients affected by oral squamous cell carcinoma and identified 217 proteins. Among the identified proteins, proteases were the most abundant and the authors stated that this methodology can be broadly adopted in the search for biomarkers. Li *et al*. studied the TIF of 20 patients suffering from non-small cell lung cancer by centrifugation. Peroxiredoxin expression was statistically elevated in the cancer group compared to patients suffering from benign lung pathology. Peroxiredoxin was associated with apoptosis inhibition and with clinical and pathological variables such as age, lymph node metastasis, and TNM classification (119).

Conclusion

In every investigation involving the preparation of samples to be analyzed, it is crucial that the samples be obtained well and consistently. This is especially true in proteomics. When tissues or cells are lysed, proteases are released or activated, initiating protein degradation through the action of proteases (120). However, the studies of Hardt *et al*. indicate that protease activity can be studied using microdialysis (118). Yet other authors have conducted their extraction of TIF by surgical means, then running the specimen through PBS incubation. Teng *et al*. have, however, demonstrated that the various TIF buffer systems did not modify protein recovery and study by LC-MS/MS (121). In order to obtain and study TIF, it is necessary to have the appropriate laboratory equipment, with personel skilled in its use, and in the standardized practices currently in use. These latter problems have been overcome by rendering many of these procedures automatic. The use of TIF as a source of biomarkers for certain kinds of cancers seems a promising and potentially very useful technique, especially for those rare tumors for which no biomarkers currently exist.

Conflicts of Interest

The Authors declare no conflicts of interest.

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