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ABSTRACT

There is growing interest in the use of human whole saliva for diagnostics and disease monitoring as an alternative to blood samples. In contrast to blood, whole saliva is a non-sterile body fluid. Proper handling and storage are required to preserve the integrity of potential biomarkers. We investigated salivary autolytic degradation using a variety of approaches. We determined inhibition of protease activities by monitoring the endogenous proteome. In addition, the stability of highly protease-susceptible proteins—histatin 5, statherin, and PRP1—was assessed. Experimental variables included (a) protease inhibitors, (b) salivary pH, (c) incubation temperatures, and (d) sample heating. A cocktail containing AEBSF, aprotinin, pancreatic trypsin inhibitor, leupeptin, antipain, and EDTA could not prevent histatin 5, statherin, or PRP1 degradation in whole saliva. Among the other treatments evaluated, short-term storage of freshly collected samples on ice was effective without interfering with the chemistry of the proteome. In conclusion, whole saliva contains a unique mixture of enzymes as evidenced from their resilience to protease inhibition. Analytical evidence on protein stability is needed to ensure the validity of salivary biomarker study outcomes. Analysis of the data presented will provide help and guidance for the use of saliva samples for diagnostic purposes.

KEY WORDS: biochemistry, biomarkers, enzymology, proteolysis, saliva, salivary diagnostics.

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Whole-saliva Proteolysis and Its Impact on Salivary Diagnostics

INTRODUCTION

Human whole saliva (WS) is a complex body fluid comprised of exocrine contributions derived from the major and minor salivary glands and a variety of non-exocrine components (Mandel, 1989). The non-exocrine components are micro-organisms, desquamated oral epithelial cells, leukocytes, and a serum-like transudate that emanates from the gingival sulcus (Goodson, 2003). The unique contribution of gingival fluid to WS provides saliva with markers that are derived from the circulation. This gingival contribution makes it feasible to exploit WS diagnostically to monitor disease biomarkers typically present in serum, albeit at different concentrations (Oppenheim, 2007; Loo *et al.*, 2010). It has been recognized that saliva is a readily available biofluid that meets the demands for non-invasive, stress-free, and inexpensive collection, offering distinctive advantages over blood collection (Mandel and Wotman, 1976). Recent evidence for saliva's diagnostic capabilities include the identification of MMP-8 and -9 and osteoprotegerin as biomarkers for periodontal disease (Ramseier *et al.*, 2009), changes in salivary inflammatory cytokine profiles in asthma exacerbations (Blicharz *et al.*, 2009), salivary detection of HIV-1 (Liu *et al.*, 2011), the correlation between salivary transcriptome markers KRAS, BMD3L2, ACRV1, and DPM1 and pancreatic cancer (Zhang *et al.*, 2010), and salivary C-reactive protein, myoglobin, and myeloperoxidase biomarkers to detect acute myocardial infarction (Florianio *et al.*, 2009). The attractiveness of using WS for diagnostic purposes, however, has frequently superseded the need to preserve the stability of this body fluid during and/or after collection. The treatment of WS samples is not a straightforward matter. The major challenges with preserving WS relate to the complexity of its composition and its inherently high proteolytic activity (Helmerhorst and Oppenheim, 2007). Consequently, WS collection and storage require special precautions to preserve its constituents, particularly any potential disease biomarkers (Crouch, 2005). Specialized protocols have been proposed for proper saliva collection (Henson and Wong, 2010). The purpose of the present investigation was to provide experimental evidence regarding the best method to maintain the integrity of proteins during saliva collection and handling. We analyzed the total proteome of WS electrophoretically and chromatographically. In addition, we used histatin 5, statherin, and proline-rich protein 1 (PRP1) as test proteins for monitoring salivary proteolysis, since these are natural salivary proteins that are quickly degraded by endogenous

proteases in WS (Castagnola *et al.*, 2004; Campese *et al.*, 2009; Helmerhorst *et al.*, 2010). The ultimate goal is to provide a protocol for optimal conditions during and after saliva collection and to improve the validity of WS biomarker studies.

MATERIALS & METHODS

Saliva Collection

Stimulated WS was obtained from five individuals in accordance with protocols approved by the Institutional Review Board at Boston University. All donors presented in good health with no signs of periodontal disease or caries. WS flow was stimulated by mastication for 10 min with ~1 g of paraffin wax (Parafilm, American National Can™, Chicago, IL, USA), yielding 10- to 12-mL sample volumes. All samples were collected in tubes placed on crushed ice (McDonald *et al.*, 2011). The WS samples from the five donors were pooled. For some experiments, aliquots of pooled WS were cleared of particulate matter, such as bacteria and mammalian-derived cells, by centrifugation at 14,000 $\times$ g for 20 min at 4°C. The supernatant is referred to as WSS.

Hydrolysis of Synthetic Enzyme Substrates and the Effects of Inhibitors

Two histatin-related enzyme substrates, benzyloxycarbonyl-Phe-His-Glu-Lys-7-amino-4-methylcoumarin (Z-FHEK-AMC) and benzyloxycarbonyl-Arg-Gly-Tyr-Arg-7-amino-4-methylcoumarin (Z-RGYR-AMC), were obtained from the American Peptide Company (Sunnyvale, CA, USA). Pooled WSS was pre-incubated for 15 min with or without 19 individual protease inhibitors (Table). Z-FHEK-AMC and Z-RGYR-AMC were subsequently added to final concentrations of 60 μ M and 30 μ M, respectively. Substrate hydrolysis was measured fluorimetrically at λ_{ex} and λ_{em} of 340 nm and 465 nm, respectively, with a Genios microtiter plate reader. Measurements were conducted every 3 min during the initial incubation period (0-15 min).

Effect of an Inhibitor Cocktail on Protein Degradation in WSS and WS

The inhibitors AEBSF, aprotinin, pancreatic trypsin inhibitor, leupeptin, antipain, and EDTA were added to WS and WSS at final concentrations of 1 mM, 8 μ M, 2 μ M, 0.5 mM, 0.8 mM, and 1 mM, respectively. Salivary protein substrates used were synthetic histatin 5 (American Peptide Company, Sunnyvale, CA, USA), statherin, or PRP1, which were both isolated from parotid secretion as described previously (Oppenheim *et al.*, 1982; Flora *et al.*, 2001). All proteins were > 90% pure as determined by gel electrophoresis and chromatography (data not shown). The final concentration of histatin 5, statherin, and PRP1 added to WS or WSS with or without inhibitor cocktail was 400 μ g/mL. After 0, 1.5, and 8.0 hrs of incubation at 37°C, 100- μ L aliquots were removed, heated for 5 min in a 100°C water bath, and analyzed by reversed-phase HPLC.

Reversed-phase High-performance Liquid Chromatography (RP-HPLC)

RP-HPLC was carried out as described previously (Helmerhorst *et al.*, 2006). The eluting histatin 5, statherin, and PRP1 were quantitated with Unipoint version 3.3 software (Gilson, Middleton, WI, USA). The percentage residual (intact) protein was calculated relative to the values obtained at $t = 0$ incubation.

Effects of pH, Cooling, and Heating on Histatin 5 Degradation in WS

To assess the effect of pH on protein stability, we adjusted WS samples to pH 3.0 or 4.0 with HCl, left them unadjusted (pH 7.2), or adjusted them with NaOH to pH 10.0. To assess the effect of temperature, we incubated the unadjusted WS sample either on ice (0°C), in the fridge (4°C), at room temperature (22°C), or in the incubator (37°C). To study the effect of heat, we placed an unadjusted WS sample in a 100°C waterbath for 10 min. Histatin 5 was added to all WS aliquots to a final concentration of 200 μ g/mL. Incubations were carried out at 37°C, except for experimental samples placed at 0, 4, and 22°C. Aliquots of 100 μ L were removed and heated after various time intervals. EDTA was added to a final concentration of 2.5 mM to complex calcium ions, and samples were dried in a Speedvac (Eppendorf, Hauppauge, NY, USA). Histatin-spiked samples were re-suspended in 20 μ L sample buffer and analyzed by cationic PAGE (Flora *et al.*, 2001) or 12% precast BisTris-PAGE (Invitrogen, Carlsbad, CA, USA).

Cationic Polyacrylamide Gel Electrophoresis (Cationic PAGE) and Histatin Quantitation

Cationic PAGE and densitometric analysis of the histatin 5 band were performed as described previously (Baum *et al.*, 1977; Flora *et al.*, 2001). The intensity of the histatin 5 band at $t = 0$ (immediately after addition to WS) was set to 100%.

RESULTS

Effectiveness of Protease Inhibitors Added to WSS

The most apparent approach to achieving protease inhibition in WS is with a cocktail of protease inhibitors. We tested 19 inhibitors using Z-FHEK-AMC and Z-RGYR-AMC as substrates and pooled WSS as the enzyme source (Appendix Table). The most effective inhibitors in this evaluation were AEBSF, aprotinin, pancreatic trypsin inhibitor, leupeptin, and antipain, all being serine protease inhibitors and displaying > 85% inhibition toward both substrates. A cocktail of these inhibitors supplemented with EDTA was tested for its efficacy to stabilize the proteome of WS and WSS over time (Figs. 1A-1D). In the absence of inhibitors, the peak patterns of WSS and WS (Figs. 1A and 1B, respectively) changed dramatically over time. Such changes are consistent with protein fragmentation. In WSS and WS samples to which the inhibitor cocktail was added, however, the peak patterns remained significantly more stable (Figs. 1C

and 1D, respectively). This enhanced stability was especially true for the WSS sample. The efficacy of the inhibitor cocktail was further evaluated in WSS and WS spiked with histatin 5. The histatin peak eluted at 40 min and was clearly discernible above the WSS and WS protein background patterns (Figs. 2E-2H). The percentage of residual histatin 5 at $t = 0$, $t = 1.5$ hrs, and $t = 8.0$ hrs was calculated from the peak heights at the various incubation time-points relative to the histatin 5 peak height at $t = 0$ (Table). The inhibitor cocktail added to WSS delayed but did not prevent histatin 5 degradation. Despite the apparent lack of inhibition in WS, the fragmentation patterns of histatin 5 in WS with and without inhibitors were not identical, indicating some inhibitory effect of the cocktail on the fragmentation process. Experiments were also carried out with statherin and PRP1 as enzyme substrates. As observed with histatin 5, both of these proteins were rapidly degraded in WSS or WS. The inhibitor cocktail reduced proteolysis rates somewhat, as evidenced from the overall higher residual amounts of histatin 5, PRP1, and statherin in WSS or WS. However, the cocktail did not prevent the degradation of any of the 3 added proteins (Table).

Effect of WS Sample pH

Given the weak inhibition by the protease inhibitor cocktail, we investigated other means to achieve inhibition of proteases in WS. It is well known that enzyme activities are pH dependent. We first investigated the effect of pH on the stability of endogenous WS proteins (non-spiked samples) over a 24 h incubation time interval. In pH-unadjusted saliva samples, proteins degraded in less than 5 h (Figure 2). Acidification of WS to pH 3.0 prevented protein degradation. The pH of the WS sample was critical since proteins were stable at pH 3.0 but not at pH 4.0. At pH 10.0, the overall protein degradation was reduced. Degradation of histatin 5 added to the same WS samples is shown in the bottom panels showing that sample acidification was effective in preventing the degradation of this protein. Subsequently, histatin degradation was monitored at more time points in WS adjusted to pH 3.0, 7.2, and 10.0 (Figure 3A) and the histatin bands were quantitated by densitometric analysis (Figure 3B). From the data shown in Figure 3B it was established that the $t_{1/2}$ values for histatin degradation were >24 h, 8 min, and 24 min, at pH 3.0, 7.2, 10.0, respectively. The data confirmed the complete inhibition of salivary proteases at pH 3.0. At pH 10.0 histatin 5 was degraded

Figure 1. Effects of inhibitor cocktails on protein degradation in WSS and WS. WSS and WS, or WSS or WS spiked with 400 µg/mL histatin 5, were incubated at 37°C. Histatin degradation was monitored by RP-HPLC. (A) WSS incubated without inhibitors; (B) WS incubated without inhibitors; (C) WSS incubated with inhibitors; (D) WS incubated with inhibitors; (E) WSS + histatin 5, incubated without inhibitors; (F) WS + histatin 5, incubated without inhibitors; (G) WSS + histatin 5, incubated with inhibitors; (H) WS + histatin 5, incubated with inhibitors.

but the overall protein banding pattern appeared relatively stable at this pH value (Figure 2).

Effect of Sample Cooling

The histatin 5 degradation kinetics at various temperatures are shown in Fig. 3C and the densitometric analysis in Fig. 3D. The $t_{1/2}$ for histatin 5 degradation in WS samples incubated at 0°C, 4°C, 22°C, and 37°C were 4 hrs 20 min, 3 hrs 10 min, 35 min, and 22 min, respectively. As expected, the lower the incubation temperature, the lower the rates of degradation. Less than 10% degradation was observed in samples stored on ice for up to 1 hr. The results indicate that placement of samples on ice does not protect protease-sensitive biomarkers indefinitely, and rapid processing of samples (e.g., centrifugation and freezing) is warranted after collection.

Table. Percentage of Residual Intact Histatin 5, Statherin, or PRP1 Added to WSS or WS without or with Inhibitor Cocktail Incubated for 0 hr, 1.5 hrs, and 8 hrs*

	Without Inhibitor Cocktail			With Inhibitor Cocktail		
	0 hr	1.5 hrs	8 hrs	0 hr	1.5 hrs	8 hrs
In WSS						
Histatin 5	100	24.8	0	100	73.8	35.2
Statherin	100	42.9	0	100	63.7	0
PRP1	100	57.9	3.9	100	81.5	54.5
In WS						
Histatin 5	100	0	0	100	10.8	0
Statherin	100	13.6	0	100	45.0	0
PRP1	100	12	0	100	60.7	37.5

*Inhibitor cocktail contained AEBSEF, aprotinin, pancreatic trypsin inhibitor, leupeptin, antipain and EDTA at final concentrations of 1 mM, 8 μ M, 2 μ M, 0.5 mM, 0.8 mM, and 1 mM, respectively.

Histatin 5, statherin, and PRP1 were added at 400 μ g/mL. Incubations were carried out at 37°C. Residual amounts of histatin 5, statherin, and PRP1 were determined from respective peak heights in samples analyzed by RP-HPLC. Data presented are from one experiment and show consistency in terms of low inhibitor efficacy in WSS as well as in WS.

Figure 2. Effect of pH adjustment on the stability of endogenous WS proteins. Stimulated WS was obtained from five individuals, pooled and either acidified to pH 3.0 or 4.0 with HCl, left unadjusted (pH 7.2), or made basic by the addition of NaOH (pH 10.0). Samples were incubated at 37°C. Protein degradation was monitored by SDS PAGE. Bottom panels: stability of histatin 5 in the same WS samples spiked with 200 μ g/mL histatin 5.

Effect of Sample Heating

The last experiment was designed to investigate if exposure to heat in a 100°C water bath could abolish WS protease activity. The results of the stability study of histatin 5 incubated at 37°C in unheated and heated WS are shown in Figs. 3E and 3F. Heat treatment resulted in a $t_{1/2}$ value of ~9 hrs as opposed to ~15 min in unboiled samples. In heated WS, histatin 5 was stable for 3 hrs (< 10% degradation). It is of interest that heating did not completely abolish all proteolytic enzymatic activities in WS, as evidenced from the very slow but continued degradation of histatin 5 in these samples.

DISCUSSION

When WS is to be used for diagnostic purposes, it is important to apply a sample treatment that prevents auto-degradation of the proteins by proteolytic enzymes. We studied conditions for minimizing or eliminating the inherent proteolytic activity of

WS. Analysis of the data obtained showed that AEBSEF, aprotinin, pancreatic trypsin inhibitor, leupeptin, and antipain each strongly inhibited hydrolysis of Z-FHEK-AMC and Z-RGYR-AMC. Based on these results, and the fact that these inhibitors are known to be effective against a broad spectrum of proteases (www.merops.org), they were mixed into a cocktail for use in WS. While this cocktail was promising in controlling WS protease activities, the results were disappointing. Interestingly, another study using a commercially available inhibitor mixture (product P8340 from Sigma [St. Louis, MO, USA], containing AEBSEF, aprotinin, bestatin, E-64, leupeptin, and pepstatin A), also showed low inhibitor efficacy in saliva (Schipper *et al.*, 2007a). The similarity of these findings with inhibitors

suggests an unusually wide range of protease activities in human saliva. More promising were the non-specific approaches of protease inhibition. Lowering as well as increasing saliva pH had clear beneficial effects on protein stability, as did sample heating. However, it was noted that sample acidification and heating caused denaturation and precipitation of some proteins (Fig. 3G). Thus, these treatments cannot be universally recommended for protease inhibition. At pH 10.0, proteases were inhibited to some extent, and no protein precipitation was observed. Lastly, cooling on ice of WS samples ensured protein stability for up to 1 hr.

Overall, among the methods evaluated to abolish proteolytic activities in WS, chemical protease inhibitors yielded the least favorable results. Another downside of using protease inhibitor cocktails is that each chemical added to WS could potentially interfere with downstream analytical biomarker assays. Considering all the possible outcomes of WS treatments prior to subjecting samples to biomarker analysis, WS collection and short-term storage of samples on ice are recommended as the

safest, most practical, and most suitable handling protocol. Subsequently, WS centrifugation is encouraged to separate the cells from the protein-containing supernatant. As we have reported previously, protein degradation in WS is 7.5-fold faster than in WSS, demonstrating that most protease activity in saliva is host cell- and/or oral bacteria-associated (Sun *et al.*, 2009). Centrifugation thus will prolong the stability of soluble WS proteins. The resulting cell sediments may be frozen or subjected to protein extraction procedures for identification of biomarkers associated with this fraction, as has been reported (Xie *et al.*, 2008). Caution should be exercised when biomarker assays are conducted at ambient temperature or at 37°C, since salivary enzyme activities will be reactivated, and this may cause loss of biomarkers during analysis. Analysis of our data reveals that, in some cases, such proteolysis can be reduced in biomarker assays which can be conducted in buffers exhibiting basic pH values. For long-term storage, the method of choice for maintaining the integrity of saliva samples has been previously shown to consist of freezing at -20°C or, even better, at -80°C (Schipper *et al.*, 2007a,b).

There is increasing evidence that WS fluid contains biomarkers amenable for the diagnosis and monitoring of local or systemic disease. This opportunity, however, can be exploited only if saliva is collected and treated in recognition of its complex composition, and, after establishment in control experiments, of the conditions which minimize proteolytically induced losses of the target biomarkers. The design of a universal saliva treatment protocol is hard to envision, when one considers the various susceptibilities of individual biomarkers to proteolytic degradation. The present work has uncovered conditions of proteolysis and means for its suppression, providing the framework for developing improved protocols for salivary diagnostics.

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REFERENCES

- Baum BJ, Bird JL, Millar DB, Longton RW (1977). Isolation and partial characterization of an histidine-rich polypeptide from parotid saliva of the monkey, *Macaca nemestrina*. *Comp Biochem Physiol A Comp Physiol* 56:115-120.
- Blicharz TM, Siqueira WL, Helmerhorst EJ, Oppenheim FG, Wexler PJ, Little FF, *et al.* (2009). Fiber-optic microsphere-based antibody array

Figure 3. Effect of pH and temperature adjustments on histatin 5 degradation in WS. Histatin 5 was added to WS and its degradation was assessed by cationic PAGE (A, C and E). The pixel intensity of the histatin band was quantitated by densitometry (B, D and F). A and B, effect of pH, C and D, effect of incubation temperature, E and F, effect of sample heating prior to incubation. G, assessment of the effect of pH adjustment and sample heating on protein solubility in WSS. WSS was pH adjusted or heated, then centrifuged. Lanes 1, 2, 3, 4 represent supernatants from pH 4.0, 7.2, 10.0 or heated WSS samples, lanes 5, 6, 7, 8 represent the corresponding WSS sediments, respectively. Experiments were carried out at least twice. Representative results are shown.

- for the analysis of inflammatory cytokines in saliva. *Anal Chem* 81:2106-2114.
- Campese M, Sun X, Bosch JA, Oppenheim FG, Helmerhorst EJ (2009). Concentration and fate of histatins and acidic proline-rich proteins in the oral environment. *Arch Oral Biol* 54:345-353.
- Castagnola M, Inzitari R, Rossetti DV, Olmi C, Cabras T, Piras V, *et al.* (2004). A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. *J Biol Chem* 279:41436-41443.
- Crouch DJ (2005). Oral fluid collection: the neglected variable in oral fluid testing. *Forensic Sci Int* 150:165-173.
- Flora B, Gusman H, Helmerhorst EJ, Troxler RF, Oppenheim FG (2001). A new method for the isolation of histatins 1, 3, and 5 from parotid secretion using zinc precipitation. *Protein Expr Purif* 23:198-206.
- Floriano PN, Christodoulides N, Miller CS, Ebersole JL, Spertus J, Rose BG, *et al.* (2009). Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: a feasibility study. *Clin Chem* 55:1530-1538.
- Goodson JM (2003). Gingival crevice fluid flow. *Periodontol* 2000 31:43-54.
- Helmerhorst EJ, Oppenheim FG (2007). Saliva: a dynamic proteome. *J Dent Res* 86:680-693.
- Helmerhorst EJ, Alagü AS, Siqueira WL, Oppenheim FG (2006). Oral fluid proteolytic effects on histatin 5 structure and function. *Arch Oral Biol* 51:1061-1070.
- Helmerhorst EJ, Traboulsi G, Salih E, Oppenheim FG (2010). Mass spectrometric identification of key proteolytic cleavage sites in statherin affecting mineral homeostasis and bacterial binding domains. *J Proteome Res* 9:5413-5421.
- Henson BS, Wong DT (2010). Collection, storage, and processing of saliva samples for downstream molecular applications. *Methods Mol Biol* 666:21-30.
- Liu C, Geva E, Mauk M, Qiu X, Abrams WR, Malamud D, *et al.* (2011). An isothermal amplification reactor with an integrated isolation membrane for point-of-care detection of infectious diseases. *Analyst* 136:2069-2076.
- Loo JA, Yan W, Ramachandran P, Wong DT (2010). Comparative human salivary and plasma proteomes. *J Dent Res* 89:1016-1023.
- Mandel ID (1989). The role of saliva in maintaining oral homeostasis. *J Am Dent Assoc* 119:298-304.
- Mandel ID, Wotman S (1976). The salivary secretions in health and disease. *Oral Sci Rev* 8:25-47.
- McDonald EE, Goldberg HA, Tabbara N, Mendes FM, Siqueira WL (2011). Histatin 1 resists proteolytic degradation when adsorbed to hydroxyapatite. *J Dent Res* 90:268-272.
- Oppenheim FG (2007). Saliva diagnostics—historical perspectives and present. In: Salivary diagnostics. Wong DT, editor. Ames, IA: Wiley-Blackwell, pp. 79-93.
- Oppenheim FG, Offner GD, Troxler RF (1982). Phosphoproteins in the parotid saliva from the subhuman primate *Macaca fascicularis*. Isolation and characterization of a proline-rich phosphoglycoprotein and the complete covalent structure of a proline-rich phosphopeptide. *J Biol Chem* 257:9271-9282.
- Ramseier CA, Kinney JS, Herr AE, Braun T, Sugai JV, Shelburne CA, *et al.* (2009). Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol* 80:436-446.
- Schipper R, Loof A, de Groot J, Harthoorn L, Dransfield E, van Heerde W (2007a). SELDI-TOF-MS of saliva: methodology and pre-treatment effects. *J Chromatogr B Analyt Technol Biomed Life Sci* 847:45-53.
- Schipper RG, Silletti E, Vingerhoeds MH (2007b). Saliva as research material: biochemical, physicochemical and practical aspects. *Arch Oral Biol* 52:1114-1135.
- Sun X, Salih E, Oppenheim FG, Helmerhorst EJ (2009). Kinetics of histatin proteolysis in whole saliva and the effect on bioactive domains with metal-binding, antifungal, and wound-healing properties. *FASEB J* 23:2691-2701.
- Xie H, Onsongo G, Popko J, de Jong EP, Cao J, Carlis JV, *et al.* (2008). Proteomics analysis of cells in whole saliva from oral cancer patients via value-added three-dimensional peptide fractionation and tandem mass spectrometry. *Mol Cell Proteomics* 7:486-498.
- Zhang L, Farrell JJ, Zhou H, Elashoff D, Akin D, Park NH, *et al.* (2010). Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology* 138:949-957.e1-7.