

University of Dundee

## Parotid saliva 1 H-NMR analysis for colon cancer metabolomics

Rovera, Angela; Hector, Mark; Anderson, Paul

*Published in:*  
Special Care in Dentistry

*DOI:*  
[10.1111/scd.12635](https://doi.org/10.1111/scd.12635)

*Publication date:*  
2022

*Document Version*  
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

*Citation for published version (APA):*

Rovera, A., Hector, M., & Anderson, P. (2022). Parotid saliva 1 H-NMR analysis for colon cancer metabolomics: A case report. *Special Care in Dentistry*, 42(1), 80-85. <https://doi.org/10.1111/scd.12635>

### General rights

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

**Title page**

**Manuscript title:**

Parotid saliva  $^1\text{H-NMR}$  analysis for colon cancer metabolomic: a case report

**Running title:**

$^1\text{H-NMR}$  analysis for colon cancer metabolomic

Angela Rovera<sup>a</sup>, Mark Hector<sup>b</sup>, Paul Anderson<sup>a</sup>

**Authors:**

<sup>a</sup> Dr Angela Rovera,

BDS, MSc, FHEA, AIEMA

Dental Physical Sciences Unit, Centre for Oral Bioengineering, Institute of Dentistry, Queen Mary University of London, London E1 4NS, United Kingdom. [a.rovera@qmul.ac.uk](mailto:a.rovera@qmul.ac.uk)

<sup>b</sup> Professor Mark Hector

BSc BDS FDS RCPS (Glasg) PhD

School of Dentistry, University of Dundee, Dundee DD1 4HN, United Kingdom.

[m.p.hector@dundee.ac.uk](mailto:m.p.hector@dundee.ac.uk)

<sup>a</sup> Professor Paul Anderson

BSc PhD MInstP CPhys

Dental Physical Sciences Unit, Centre for Oral Bioengineering, Institute of Dentistry, Queen Mary University of London, London E1 4NS, United Kingdom. [p.anderson@qmul.ac.uk](mailto:p.anderson@qmul.ac.uk)

Corresponding author:

Angela Rovera

Postal Address: Dental Physical Sciences Unit, 2nd Fl. Bancroft Building, Queen Mary University of London, Mile End Road, London, E1 4NS

+44 20 7882 5974 (Phone)

+44 20 7882 7979 (Fax)

E-mail: [a.rovera@qmul.ac.uk](mailto:a.rovera@qmul.ac.uk)

## **Abstract**

*Background:* A key priority in colon cancer research is the identification of molecular biomarkers to improve early diagnosis, guide prognosis, and the design of new therapeutic approaches. Saliva is a powerful diagnostic biofluid that can be used to detect systemic alterations. This study aimed to investigate the parotid saliva (PS) metabolic Proton Nuclear Magnetic Resonance ( $^1\text{H-NMR}$ ) profile of a patient diagnosed with colon cancer, and the subsequent changes one year after the end of chemotherapy.

*Case report:* We describe the  $^1\text{H-NMR}$  PS spectrum of a 65-year-old woman diagnosed with colon cancer (G3 pT3 pN1c) (T0), and the changes in the spectrum from PS collected one year after the end of chemotherapy (XELOX: capecitabine plus oxaliplatin) (T1). The data was co-analysed with blood test cancer antigens (S-CEA; S-CA19-9) and thyroid peroxidase antibody (TPOAb) measurements obtained simultaneously in order to identify peaks and interpret the spectra. The blood cancer antigens (S-CEA; S-CA19-9) and the PS  $^1\text{H-NMR}$  peaks for fatty acids, lactate, acetate, N-acetyl sugars, citrate, tyrosine, saccharides, and formate decreased at T1 compared to T0. Whereas, the thyroid peroxidase antibody (TPOAb) blood values increased at T1 compared to T0 reflecting the changes in the  $^1\text{H-NMR}$  spectral window of 1- 3.5 ppm.

*Conclusion:* PS  $^1\text{H-NMR}$  profiling identified modified metabolites that revealed cancer cells metabolism disturbances that subsequently decreased with time throughout treatment. These altered metabolites are potential biomarkers, providing a molecular diagnostic approach for clinical diagnosis and prognosis of human colon cancer.

**Keywords:** saliva,  $^1\text{H-NMR}$  analysis, colon cancer

## 1. Introduction

Colorectal Cancer (CRC) incidence and mortality rates vary markedly around the world. Globally, CRC is the third most commonly diagnosed cancer in males and the second in females, with 1.8 million new cases and almost 861,000 deaths in 2018 according to the World Health Organization GLOBOCAN database ([www.gco.iacr.fr](http://www.gco.iacr.fr)).

Although colonoscopy remains the gold standard for diagnosing precancerous lesions and CRC, this approach is invasive, expensive, and uncomfortable, therefore precluding it as a cost-effective population-based screening test.<sup>1</sup> Fecal occult blood testing (FOBT) and blood cancer biomarkers, including carcinoembryonic antigen (CEA), carbohydrate antigen (CA19-9) are used clinically, but have relatively low sensitivities and specificities.<sup>1</sup> A key priority in cancer research is therefore the identification of molecular biomarkers to improve early diagnosis, guide prognosis and design new therapeutic approaches.

Over the last decade, salivary diagnostics has drawn significant attention for the detection of specific biomarkers. One reason is that sample collection and processing are simple, cost-effective, precise, and does not cause patient discomfort.<sup>2</sup> Unlike colonoscopy and CT colonography, saliva collection does not require a specialised practitioner and complex patient's preparation procedures, e.g. bowel preparation.

Saliva metabolomics enables measuring levels of endogenous metabolites, thus facilitating biomarker discovery and monitoring during disease progression using a broad collection of technologies.<sup>3 4</sup> The proposed mechanisms by which distal cancers mediate changes in salivary biomarker profiles and advances in saliva used for systemic cancer detection are well-reported in the literature.<sup>3</sup>

Metabolic profiling of saliva using Proton Nuclear Magnetic Resonance spectroscopy ( $^1\text{H-NMR}$ ) has demonstrated high sensitivity for biomarker identification <sup>2</sup> and reproducibility for components investigation with as little as 450  $\mu\text{l}$  of saliva without extensive sample preparation.<sup>5</sup>  $^1\text{H-NMR}$  spectroscopy probes the chemical environment of all the proton nuclei within saliva and records the nuclear spin electromagnetic radiation resulting from rapid magnetic field changes aroused in the external magnetic field.<sup>5</sup> A  $^1\text{H-NMR}$  spectrum is a plot of the applied radiofrequency against absorption where the signal is referred to as a resonance, and its frequency is the “chemical shift” expressed in parts per million (ppm). If a saliva component has changed chemically, the corresponding  $^1\text{H-NMR}$  peaks will shift their position, line shape, or even disappear, due to the changed local chemical environment.<sup>6</sup>

$^1\text{H-NMR}$  studies of saliva have previously analysed whole-mouth saliva (WMS).<sup>2</sup> WMS is a complex fluid derived from the three major paired salivary glands, and thousands of minor glands distributed throughout the oral cavity. It also contains bacteria, as well as host-derived cells.<sup>7</sup> This cellular content of WMS can obscure the analysis because of the ongoing metabolic activity of oral bacteria, and neutrophils.<sup>8</sup> However, this issue can be eliminated by the aseptic collection of saliva directly from a single salivary gland. This is most commonly achieved via the parotid salivary gland, where if collected appropriately, the saliva is therefore sterile.<sup>9</sup> There have been no studies on metabolic analysis of parotid saliva (PS) by  $^1\text{H-NMR}$  to identify colon cancer biomarkers.

This study reports the PS metabolic  $^1\text{H-NMR}$  profile of a patient diagnosed with colon cancer, and the subsequent changes one year after the end of chemotherapy. The data is co-analysed with blood test antibody measurements taken at the same time in order to identify peaks and interpret the spectra.

## **2. Case report**

This study was carried under the ethical principles of Good Clinical Practice and the Declaration of Helsinki following approval from the Local Ethics Committee “ASO Santa Croce e Carle” Cuneo, Italy (n.66-17 of 10/05/2017). The subject signed a written informed consent form before participating in the study.

### **2.1 Case description**

We present a case of a 65-year-old woman, who was diagnosed with colon cancer in 2018. The non-smoking patient was not taking any medication nor was under any treatment for other concomitant pathologies. She did not have any previous medical or surgical history. She reported occasional abdominal pain. Preliminary investigation with fecal occult blood revealed positive results. Initial blood test showed abnormal values of hemoglobin 8.5 g/dL (normal range 12-16), hematocrit 30.2% (normal range 37- 47), S-CEA 10.3 ng/mL (normal value up to 5), and S-CA19-9 71.7 U/mL (normal up to 37) (Table 1). Colonoscopy, CT scan and intraoperative histological exam confirmed the colon cancer at stage G3 pT3 pN1c (pT3 N1c). Surgical treatment including subtotal colectomy and anastomosis were completed at the same time. Postoperative adjuvant chemotherapy was administered for 12 months. This included the combination of capecitabine with oxaliplatin (XELOX). The follow-up included blood test every six months, CT scan and colonoscopy performed alternately every six months in order to carefully monitor the patient for the 5 years post-treatment.

### **2.2 Sample Collection and Preparation**

PS samples were collected from the patient at the same time as the first blood test for cancer diagnosis (T0). A parotid further sample was collected by the same dentist one year after the

end of the chemotherapy, again at the same time as the follow-up blood test (T1). The patient was asked not to eat or drink except water, nor perform oral hygiene practices and not to undergo heavy physical stress, at least two hours before saliva collection.<sup>5</sup>

Unilateral stimulated PS samples were collected using a Lashley cup placed over the Stenson's duct, which drains saliva into the oral cavity.<sup>10</sup> The patient was required to chew paraffin wax throughout the collection.<sup>5</sup> A one-minute pre-sampling period was recorded, then, 5 ml of PS was collected into sterilized low-affinity conical plastic collection tubes (Fisher Scientific, UK) under 1.0 ml of paraffin oil in order to avoid CO<sub>2</sub> loss.<sup>5</sup> The collection time was fixed between 9 and 11 am.<sup>5</sup>

All saliva tubes were kept on ice from the moment of collection, and immediately frozen (-35 °C). Subsequently, the PS samples were thawed immediately prior to <sup>1</sup>H-NMR analysis. Recent findings<sup>11</sup> indicate that saliva components are resilient to freezing.

### **2.3 <sup>1</sup>H-NMR Spectroscopy Sample Preparation and Analysis**

Aliquots (600 µL) of each PS sample were filtered using a glass wool pipette and then mixed with Deuterium Oxide 10% (D<sub>2</sub>O) (deuterium oxide, D, 99.9% Cambridge Isotope Laboratories, USA) as a lock signal. Saliva <sup>1</sup>H solution NMR spectra were acquired using a Bruker 600 MHz spectrometer (Bruker Biospin, Karlsruhe, Germany) in Fourier Transform mode, with the sample temperature controlled at 26°C. The <sup>1</sup>H-NMR spectra were processed using Bruker TopSpin software (Bruker Biospin, Rheinstetten, Germany, version 4.0.5). The chemical shift of each peak was assigned chemically (in part per million (ppm)) based on literature<sup>12</sup> and Human Metabolome Database (HMDB) (<http://www.hmdb.ca>) (Table 1).

### 3. Results

Figure 1 shows the <sup>1</sup>H-NMR spectral window from 0 to 10 ppm at T0 (a) and T1 (b).

Figure 1a (T0) depicts high peaks in the region of 0- 5 ppm corresponding to fatty acids (FAs), lactate, acetate, acetamido methyl groups of N-acetyl sugars (N-Ac), citrate, tyrosine (Tyr), and saccharides region referring to the chemical shift assignment, all identified in Table 1. Figure 1b (T1) shows a reduction in these peak intensities of the chemical shifts corresponding to FAs, lactate, acetate, N-Ac, citrate, Tyr, and saccharide region. Further, there is an increase in three peaks of the chemical shift of 3.5, 2, and 1 ppm compared to the similar region at T0.

Figure 1a (T0) shows a substantial isolated peak at 8.5 ppm. This peak has previously been ascribed to the formate anion chemical shift.<sup>2</sup> However, Figure 1b (T1) shows the peak intensity at 8.5 ppm is substantially decreased.

Table 2 shows the blood test values at T0 and T1. At T0, values of hemoglobin, and hematocrit decrease below the normal range, whereas the blood cancer biomarkers (S-CEA and S-CA19-9) dramatically increase above the threshold. At T1, S-CEA and S-CA19-9 values decrease to the normal range, while the thyroid peroxidase antibody (TPOAb) values rise from T0 to T1.

### 4. Discussion

Cancer cells, unlike normal cells, have the capacity to modify cellular metabolic processes with the final purpose to survive despite scarce resources to growth.<sup>13</sup>



This report identifies the up-regulation of fatty acids, lactate, acetate, acetamido methyl groups of N-acetyl sugars, citrate, tyrosine, saccharides, and formate at T0 compared to T1.

The upregulation of PS fatty acids in the CRC patient at T0 is consistent with a previous study indicating that lipolysis and FA oxidation are dominant bioenergetic pathways in CRC tissue.<sup>14</sup>

The upregulation PS lactate at T0 reflects its accumulation in CRC tissues. This is consistent with previous reports observing an increase of lactate in oral cancer, stomach cancer, and CRC tissues.<sup>15</sup> Cancer cells increase glucose uptake to satisfy the energy demand for sustaining their rapid proliferation, and consequently lactate accumulate in cancer tissues.<sup>15</sup> Further, lactate can make the extracellular pH strongly acidic which stimulate cancer metastasis.

The upregulation PS acetate at T0 supports previous <sup>1</sup>H-NMR studies observing that acetate increases in oesophageal cancer and CRC tissues which impacts on the resistance of cancer cells and therefore the survival of patients.<sup>15,16</sup>

The upregulation of PS acetamido methyl groups of N-acetyl sugars and saccharides at T0 confirms that the altered CRC cell metabolism increases glycoconjugates level following cancer progression.

PS citrate is upregulated at T0 compared to T1. Normally, when sufficient citrate is produced by the tricarboxylic acid (TCA) cycle, it causes unfavourable feedback on glycolysis (Pasteur effect), and on the TCA cycle, decreasing or arresting these pathways, whereas, it stimulates gluconeogenesis and ATP-consuming lipid synthesis.<sup>17</sup>

The upregulation of PS tyrosine at T0 confirms that CRC tissues have significant accumulation of amino acids indicating an increased protein catabolism (autophagic breakdown).<sup>15</sup>

The upregulation of PS formate at T0 supports previous <sup>1</sup>H-NMR studies observing increased formate peaks in CRC<sup>15</sup>, and human oesophageal cancer<sup>16</sup>, in order to meet the cancer cells' requirements for single-carbon groups nucleotide (purine and thymidylate) synthesis, as well as for the provision of methyl groups for methylation reactions. The results shown here demonstrate a downregulation of PS formate at T1 confirming that the inhibition of mitochondrial formate production by genetic or pharmacological interventions reduces formate overflow, which can inhibit cancer cell proliferation.<sup>18</sup>

Treatment for CRC including fluoropyrimidine-based chemotherapy, with or without bevacizumab, has also been demonstrated to cause thyroiditis.<sup>19</sup> Table 2 indicates that at T1, the patient had elevated antithyroid peroxidase antibodies (TPOAb). Autoimmune changes that occur in endocrine glands (e.g., thyroid) may also occur in salivary glands. This explains the <sup>1</sup>H-NMR spectrum variation in the spectral window 1- 3.5 ppm at T1.

## **5. Conclusion**

The PS <sup>1</sup>H-NMR profile showed modified metabolites that revealed metabolism disturbances in CRC cells, that subsequently decreased with time throughout treatment.

The blood cancer antigens (S-CEA; S-CA19-9) and the PS <sup>1</sup>H-NMR peaks for fatty acids, lactate, acetate, N-acetyl sugars, citrate, tyrosine, saccharides, and formate decreased from T0 to T1. Whereas, the thyroid peroxidase antibody (TPOAb) blood values increased from T0 to T1 reflecting the changes in the <sup>1</sup>H-NMR spectral window of 1- 3.5 ppm. All these altered metabolites are potential biomarkers, providing a promising molecular diagnostic approach for clinical diagnosis and prognosis of human colon cancer. It is recommended that further research should be undertaken on large scale diagnostic accuracy study to validate the assessed diagnostic method. Saliva metabolomics is an effective, non-invasive screening tool to facilitate CRC diagnosis, as PS collection and processing are simple, effective, precise and cause minimum discomfort to the examined patient.

## 6. References

1. Tanaka T, Tanaka M, Tanaka T, Ishigamori R. Biomarkers for colorectal cancer. Vol. 11, International Journal of Molecular Sciences. Multidisciplinary Digital Publishing Institute (MDPI); 2010. p. 3209–25.
2. Gardner A, Carpenter G, So PW. Salivary metabolomics: From diagnostic biomarker discovery to investigating biological function. Vol. 10, Metabolites. MDPI AG; 2020. p. 47.
3. Wang X, Kaczor-Urbanowicz KE, Wong DTW. Salivary biomarkers in cancer detection. Vol. 34, Medical Oncology. Humana Press Inc.; 2017. p. 7.
4. Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics*. 2010 Mar 10;6(1):78–95.
5. Rovera A, Rovera G, Alzahrani A, Hector M, Anderson P. Correlation between parotid saliva composition and dental caries using <sup>31</sup>P-NMR and ICDAS score. *Arch Oral Biol*. 2020 Mar 1;111.
6. Zuiderweg ERP. Mapping protein-protein interactions in solution by NMR spectroscopy. *Biochemistry*. 2002 Jan;41(1):1–7.
7. Gardner A, Parkes HG, So PW, Carpenter GH. Determining bacterial and host contributions to the human salivary metabolome. *J Oral Microbiol*. 2019 Jan 1;11(1):1617014.
8. Helmerhorst EJ, Dawes C, Oppenheim FG. The complexity of oral physiology and its impact on salivary diagnostics. *Oral Dis*. 2018 Apr 1;24(3):363–71.
9. Schrøder SA, Bardow A, Eickhardt-Dalbøge S, Johansen HK, Homøe P. Is parotid saliva sterile on entry to the oral cavity? *Acta Otolaryngol*. 2017 Jul 3;137(7):762–4.
10. Lashley KS. Reflex secretion of the human parotid gland. *J Exp Psychol*.

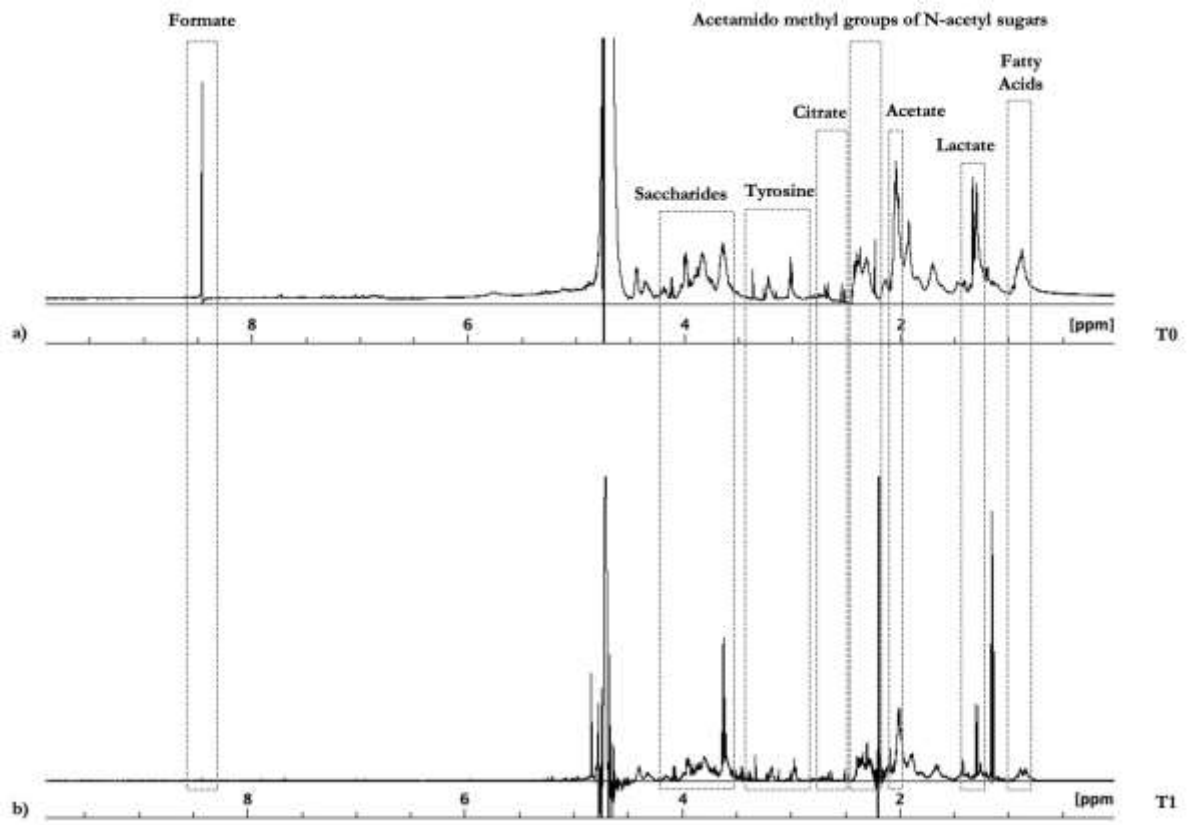
- 1916;1(6):461–93.
11. Gardner A, Parkes HG, Carpenter GH, So PW. Developing and Standardizing a Protocol for Quantitative Proton Nuclear Magnetic Resonance ( $^1\text{H}$  NMR) Spectroscopy of Saliva. *J Proteome Res.* 2018 Apr 6;17(4):1521–31.
  12. Silwood CJL, Lynch E, Claxson AWD, Grootveld MC.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopic analysis of human saliva. *J Dent Res.* 2002 Jun;81(6):422–7.
  13. Schulze A, Harris AL. How cancer metabolism is tuned for proliferation and vulnerable to disruption. Vol. 491, *Nature*. Nature Publishing Group; 2012. p. 364–73.
  14. Tian Y, Xu T, Huang J, Zhang L, Xu S, Xiong B, et al. Tissue metabonomic phenotyping for diagnosis and prognosis of human colorectal cancer. *Sci Rep.* 2016 Feb 15;6.
  15. Wang H, Wang L, Zhang H, Deng P, Chen J, Zhou B, et al.  $^1\text{H}$  NMR-based metabolic profiling of human rectal cancer tissue. *Mol Cancer.* 2013 Oct 18;12(1):121.
  16. Wang L, Chen J, Chen L, Deng P, Bu Q, Xiang P, et al.  $^1\text{H}$ -NMR based metabonomic profiling of human esophageal cancer tissue. *Mol Cancer.* 2013 Apr 4;12(1):25.
  17. Icard P, Poulain L, Lincet H. Understanding the central role of citrate in the metabolism of cancer cells. Vol. 1825, *Biochimica et Biophysica Acta - Reviews on Cancer*. *Biochim Biophys Acta*; 2012. p. 111–6.
  18. Pietzke M, Meiser J, Vazquez A. Formate metabolism in health and disease. Vol. 33, *Molecular Metabolism*. Elsevier GmbH; 2020. p. 23–37.
  19. Fujiwara Y, Chayahara N, Mukohara T, Kiyota N, Tomioka H, Funakoshi Y, et al. Hypothyroidism in patients with colorectal carcinoma treated with fluoropyrimidines. *Oncol Rep.* 2013 Oct;30(4):1802–6.

**Table 1.** <sup>1</sup>H-NMR components assignment for human saliva based on literature and Human Metabolome Database (HMDB) at 600 MHz.

<b>Metabolite (HMDB number)</b>	<b>Chemical shift (ppm), and multiplicity of characteristic resonances</b>	<b>Assignment</b>
Acetate (HMDB0000042)	1.92, singlet	CH3
Citrate (HMDB0000094)	2.51, doublet	CH2
	2.67, doublet	CH2
Fatty Acid	0.90, multiplet	CH3
	1.18 multiplet	CH2
Formate (HMDB0000142)	8.45, singlet	CH
Lactate (HMDB0000190)	1.33, doublet	CH2
	4.1, quartet	
Acetamido methyl groups of N-acetyl sugars	2.05 multiplet	CH3
Phenylalanine (HMDB0000159)	3.19, multiplet	CH2
	3.98, multiplet	CH
	7.32, doublet	H2, 2'
	7.36, multiplet	H3, 3'
	7.42, multiplet	H4
Tyrosine (HMDB0000158)	3.02, multiplet	CH2
	3.17, multiplet	CH2
	3.92, multiplet	CH
	6.88, multiplet	H2, 2'
	7.17, multiplet	H3, 3'

**Table 2.** Blood test values at the time of colon cancer diagnosis (T0) and 1 year after the end of the chemotherapy (T1).

	<b>T0</b>	<b>T1</b>	<b>Normal range values</b>
<b>Hemoglobin</b>	8.5 g/dL	15.2 g/dL	12-16 g/dL
<b>Hematocrit</b>	30.2 %	46.0 %	37- 47 %
<b>S-CEA (carcinoma embryonic antigen)</b>	10.3 ng/mL	<0.5 ng/mL	up to 5 ng/mL
<b>S-CA19-9 (carbohydrate antigen)</b>	71.7 U/mL	11.0 U/mL	up to 37 U/mL
<b>TPOAb (thyroid peroxidase antibody)</b>	40 U/mL	201 U/mL	up to 60 U/mL



**Figure 1.** Parotid saliva 600 MHz  $^1\text{H}$  NMR spectra at T0 (a) and T1 (b).