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Keywords: oral squamous cell carcinoma, biomarkers, Matrix-Assisted Laser Desoption/Ionization Time-of-Flight, zinc finger protein 510, ClinProt

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Conclusions: Identifying 24-mer ZNF510 peptide as OSCC-related salivary biomarkers via proteomic approach proved useful in adjunct diagnosis for early detection rather than specific diagnosis marker for progression of OSCC patients.

Dear Editor,

We would like to submit our manuscript entitled "**Salivary zinc finger protein 510 peptide as a novel biomarker for detection of oral squamous cell carcinoma in early stages**" to Clinica Chimica Acta.

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Thanks for your helps.

Sincerely,

Cheng-Wen Lin, PhD
Professor
Department of Medical Laboratory Science and Biotechnology
China Medical University

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Corresponding Author: Cheng-Wen Lin

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2011/01/07

Salivary zinc finger protein 510 peptide as a novel biomarker for detection of oral squamous cell carcinoma in early stages

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Running title: Salivary ZNF510 as an OSCC biomarker

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54 **Key words:** oral squamous cell carcinoma, biomarkers, Matrix-Assisted Laser
55 Desoption/Ionization Time-of-Flight, zinc finger protein 510, ClinProt

56

57 **Abbreviations:** AUC, area under ROC curve; ELISA, Enzyme-linked immunosorbent assay;
58 MALDI-TOF, Matrix-Assisted Laser Desoption/Ionization Time-of-Flight; MS, Mass
59 Spectrometry; OSCC, oral squamous cell carcinoma; PCA, principal component analysis;
60 ROC, Receiver-Operating Characteristic.

61

1. Introduction

Oral cancer composes 2-3% of all malignancies [1], with over 300,000 cases newly diagnosed yearly worldwide [2]. Over 90% of these cases are categorized as oral squamous cell carcinoma (OSCC) with a high degree of local invasiveness and high rate of metastasis, which leads to its high mortality [1-3]. Prevalence of oral cancer is markedly higher in Asia, compared with other industrialized nations [4-7]. In Taiwan, it is the fourth most common cause of death from cancer in males, after liver, lung and colorectal cancer (Department of Health, Executive Yuan, Taiwan, 2006) [8]. Common risk factors for oral cancer include tobacco, alcohol and chewing betel quid. The risks are synergistic and might result in large areas of mucosal change or stimulate carcinogenesis in the oral cavity [6].

Oral cancer progresses from premalignant lesions to serial histological and clinical changes [9]. Clinical examination of oral cancer includes a thorough head, neck and intraoral visual examination and palpation of the oral cavity. Although the oral cavity is amenable to direct examination, oral cancer is often not detected until a late stage [10]. Biopsy of suspicious lesions remains the standard method to determine their nature. Besides discomfort associated with invasive biopsy, non-uniform appearance of (pre)cancerous lesions may hamper determination of site for biopsy, crucial in histopathological verification of oral cancer. Even with significant advances in treatment modalities (surgery, radiotherapy, chemotherapy or combination thereof), overall five-year survival rate for oral cancer hovers

around fifty percent [11-13]. New screening tumor markers are vital to improving identification of early malignant oral lesions, especially in at-risk populations, yet there is no effective means of accurate and feasible mass screening. Since it is heterogeneous, there apparently exist multiple cellular pathways in progress of tumorigenesis. We attempted to identify useful biomarkers for rapid and accurate diagnosis of saliva samples that also can differentiate stages of oral cancer progression.

In case of oral cancer, saliva is a good candidate for analysis to identify biomarkers [14]. Whole saliva consists of secretions from major and minor salivary glands and gingival crevicular fluid [15]. Compared with blood sampling or biopsy, use of saliva for oral cancer screening holds advantages: e.g., easier, less invasive, better tolerated by patients. Significant increase of salivary soluble CD44 (solCD44) levels has been identified in head and neck squamous cell carcinoma (HNSCC) patients versus normal controls [16]. Comprehensive analysis of salivary parameters shows that secretory immunoglobulin A, 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) are lower, while insulin growth factor I, metalloproteinases MMP-9, carbonyls and Cyclin D1 (CycD1) are higher in OSCC patients [17,18]. Recent proteomic analysis of saliva samples from OSCC patients and matched healthy subjects, using capillary reversed-phase liquid chromatography with quadruple time-of-flight (LC-Q-TOF) mass spectrometry, indicated five candidate OSCC biomarkers (M2BP, MRP14, CD59, profilin, and catalase), successfully

validated by immunoassays on an independent set of OSCC patients and matched healthy subjects [19]. Still, these candidate OSCC biomarkers have no significant correlation with tumor size, stage and recurrence.

C8-magnetic bead and mass spectrum (C_8/MS) have been applied to this approach in human diseases [20]. We thus intended to identify salivary biomarkers of early stages of OSCC, using C_8/MS and *ClinProTools* software. Potential salivary peptides as OSCC biomarkers were then subjected to validation by competitive binding inhibition and direct binding ELISA analysis of all samples from OSCC patients and control individuals. We identified significant association of linear increase in salivary ZNF510 peptide levels with tumor progression of OSCC.

2. Materials and methods

2.1. Human subjects

The 71 persons who enrolled in this study with or without OSCC were treated at China Medical University Hospital (Taichung, Taiwan) from February 2007 to November 2008. Ages ranged from 21 to 78 years (mean±standard deviation, 53.3 ± 11.5 years); 62 subjects (87.3%) were male. Saliva collection protocol was approved by the Institutional Review Board of China Medical University Hospital (permission number DMR96-IRB-80). Subjects provided informed consent information prior to experimental protocol. Clinical staging for

OSCC patients was reviewed according to the universal TNM staging system of the International Union against Cancer (UICC): tumor size (T), nodal metastasis (N) and distant metastasis (M) [21]. The OSCC group consisted of 41 patients with a mean age of 49.8 ± 11.2 (39 male, 2 female): T1 = 17, T2 = 15, T3 = 4, and T4 = 5 (Supplemental Table 1); control group included 30 subjects (mean age, 44.9 ± 10.1; 23 male, 7 female) without oral cancer. Samples were collected from patients and controls, then stored at −80°C until analysis. Diagnosis was based on clinical examination and verified by pathological examination via biopsy. Inclusion criteria for all subjects were 20+ years of age and immunocompetent. Exclusion criteria for OSCC patients were having no history of chemotherapy, irradiation, immunocompromise or lack of proper consent. For controls, systemic conditions associated with immune dysfunction like diabetes, previous chemotherapy, irradiation, and/or presence of oral mucosal lesions, pregnancy or lactation were criteria for exclusion.

2.2. Sample collection

Subjects were requested to quit smoking tobacco, drinking alcohol and/or chewing betel nut at least 24 hours before saliva collection; protocol was performed in the early morning. The day before collection, patients were instructed to brush their teeth and rinse their mouths by gargling with clean water before sleeping. They were prohibited to eat, drink or brush their teeth for one hour before salivary collection. When starting expectoration, all were instructed

138 to gargle and rinse with normal saline and swallow the first bolus of saliva only. Samples
139 were spit into collecting cups, without mechanical or chemical stimulation. Complete
140 Protease Inhibitor Cocktail (Roche, Mannheim, Germany) was added, following
141 manufacturer's protocol and transferred to a microcentrifuge tube for further centrifuge at
142 16,000 × *g* at 4°C for 10 min. Supernatant were collected and stored at –80°C until analysis.
143
144 *2.3. Proteomics analysis*
145 Salivary samples collected from the oral cancer (n=41) and control groups (n=30) were
146 analyzed using C8-magnetic analysis following manufactory manual protocol (ClinProTools,
147 Bruker Daltonics, Bremen, Germany). Briefly, salivary peptides were separated using reagent
148 set with C8-magenetic beads. Measured volume of 5 µl of bead suspension was transferred to
149 a tube containing an aliquot 5 µl of saliva, and 10 µl of binding buffer, C8-magnetic beads,
150 saliva and binding buffer were mixed by pipeting up/down for 1 min. A minimal volume of
151 50% elution solvent (v/v) was added to the bead pellet and mixed for 1 min. Peptide
152 binding-beads were pulled to the side and a fraction of the elute transferred to another tube.
153 For MALDI-TOF MS sample preparation, a measured volume of pre-made matrix solution
154 containing 0.6 mg/mL α-cyano-4-hydroxycinnamic acid was added to the elute, followed by
155 mixing and transfer to the MALDI target (AnchorChipTM; Bruker Daltonics). Mass spectra
156 were further scrutinized using FlexAnalysis software (Bruker Daltonics). Peaks obtained

157 from 30 controls, 32 patients with T1 or T2 stage (T1+T2) and 9 patients with T3 or T4 stage
158 (T3+T4) were graphed as columns representing which peak intensities were normalized to the
159 total ion current of *m*/*z* between 1000 and 10000.
160
161 *2.4. Preparation of rabbit anti-ZNF510 peptide sera*
162 To validate 24-mer ZNF510 peptide level, saliva samples from OSCC patients (Stages
163 T1-T4) and control subjects were quantified by sandwich ELISA with rabbit anti-24-mer
164 ZNF510 peptide antisera. The 24-mer ZNF510 peptide was synthesized using automated
165 solid-phase synthesis (MDBio, Inc., Taipei, Taiwan). Rabbits were initially immunized with
166 500 μg of the peptide in complete Freund's adjuvant by intranodal injection and then by
167 subcutaneous injection with 500 μg in Freund's incomplete adjuvant on Day 21. Rabbits
168 were continuously boosted at 10-day intervals with 500 μg of antigen in Freund's incomplete
169 adjuvant. Anti-ZNF510 peptide sera were collected on Day 61 after the fifth injection. For
170 determining titers of the anti-ZNF510 peptide sera by ELISA, 96 wells of a microtiter plate
171 were coated with 25 μg of synthesized 24-mer ZNF510 peptides, and then incubated at 4 °C
172 overnight. Following incubation and subsequent layer of ELISA, wells were washed three
173 times with Tris-buffered saline (TBS, 50mM Tris-Hcl, 137 mM NaCl, pH 7.5) containing
174 0.05% (w/v) Tween 20 (TBST). After blocking with 5% (w/v) skim milk in PBST, 10-fold
175 serial dilutions of rabbit anti-ZNF510 peptide sera were incubated in ZNF510 peptide-coated

wells for two hours. Bound antibody was detected by HRP-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) and quantified by gauging optical density at 450 nm (OD_{450}) after development with 2,2'-azinobis (3-ethylbenthiazoline-6-sulfonate) (ABTS) and hydrogen peroxide substrates. To gauge binding ability of anti-ZNF510 peptide sera, microtiter-plate wells were coated with 10-fold serial dilutions of synthesized ZNF510 peptides ranging from 25 μg to 0.25 ng and reacted with 1:1000 and 1:10000 dilutions of rabbit anti-ZNF510 peptide sera.

2.5. Competitive and direct binding ELISA for analyzing ZNF510 peptide levels in salivary samples

For competitive binding ELISA, 96 wells of a microtiter plate were coated with 25 μg of salivary mixtures and then incubated at 4 °C overnight. Following incubation and subsequent layer of ELISA, wells were washed three times with Tris-buffered saline (TBS, 50mM Tris-Hcl, 137 mM NaCl, pH 7.5) containing 0.05% (w/v) Tween 20 (TBST). After blocking with 5% (w/v) skim milk in PBST, 1:1000 dilution of rabbit anti-ZNF510 peptide sera was incubated with or without 25 μg of synthesized 24-mer ZNF510 peptides at 37 °C for 1 h, then these mixtures were added into salivary mixture-coated wells for 2-h incubation. Bound antibody was detected by HRP-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) and quantified by gauging optical density at 405 nm (OD_{405}) after developing with 2,2'-azinobis

195 (3-ethylbenthiazoline-6-sulfonate) (ABTS) and hydrogen peroxide substrates. For direct
196 binding ELISA, 1:1000 dilution of anti-ZNF510 peptide sera were directly added into
197 salivary mixture-coated wells for 2-h incubation.
198

199 *2.6. Immunohistochemical staining of OSCC and OSCC-free tissues*

200 Two OSCC tissues from T2-stage patients and other two OSCC-free control tissues
201 were analyzed for immunohistochemical staining. Tissue sections were deparaffined and
202 rehydrated. After blocking with 1% normal goat serum, 1: 2000 dilution of rabbit
203 anti-ZNF510 peptide sera was added to the tissue sections and incubated for 1 hour, followed
204 by HRP-conjugated anti-rabbit IgG antibodies and then 3,3'-diaminobenzidine
205 (Sigma-Aldrich) as substrate.
206

207 *2.7. Statistical evaluation*

208 Statistical analysis and discriminant pattern recognition were analyzed by ClinProTools
209 2.0 software package (Bruker Daltonics), as described in manual protocol [22]. Each
210 spectrum obtained from MALDI-TOF MS with FlexAnalysis to detect peak intensities of
211 interests and performed to compile peaks across the spectra obtained from all samples.
212 Clusters of signals distinguishing between populations, based on data of healthy controls and
213 OSCC patients, were selected by QuickClassifier algorithm (QC) and Support Vector

214 Machines (SVM), following manual protocol (Bruker Daltonics). Analysis of ROC curve and
215 calculations of area under curve (AUC) were performed to examine diagnostic efficacy for
216 the 24-mer ZNF510 peptide marker. ZNF510 peptide levels in saliva between patients with
217 OSCC and healthy controls were analyzed by *Chi*-square test with Yates's correction or by
218 ANOVA analysis using SPSS program (version 10.1, SPSS Inc., IL, USA). A *P* value less
219 than 0.01 indicated statistical relevance. Group means were compared using Student *t*-test; *P*
220 value less than 0.05 was considered statistically significant.
221
222 **3. Results**
223 *3.1. Identification of biomarkers in OSCC saliva*
224 To investigate potential biomarkers, we collected saliva samples from OSCC patients
225 and OSCC-free control subjects. A total of 41 OSCC patients were recruited in this study, 39
226 (95%) males and 2 (5%) females. The age range of OSCC patients was 29 to 79 with an
227 average age of 51.2 years (Supplemental Table 1). Of 30 OSCC-free control subjects, 23
228 (76.6%) were male and 7 (23.4%) were female. The age range of OSCC-free control subjects
229 was 29 to 66 with an average age of 44.9 years. Although no significant differences were
230 detected with respect to age or gender distribution, males were more common in the OSCC
231 group. Among these 41 patients, 21 (51.2%) had buccal carcinomas, 19 (46.3%) had tongue
232 carcinomas and one patient had mouth floor cancer. Moderately differentiated keratinizing

233 squamous cell carcinoma was the most common histopathological appearance (28.8%). The
234 41 patients were classified into four categories: T1 (41.5%), T2 (36.6%), T3 (9.8%) and T4
235 (12.1%) according to UICC TNM staging [21]. Salivary peptides and proteins were purified
236 using C8-coated magnetic beads, then characterized by MALDI-TOF mass spectrometry.
237 Peptide mass fingerprint (PMF) profiles of OSCC patients (Fig. 1A) and controls (Fig 1B)
238 showed the high complexity of spectra peaks. The obtained peaks from 30 controls, 32
239 patients with T1 or T2 stage (T1+T2) and 9 patients with T3 or T4 stage (T3+T4) were
240 graphed as columns representing normalized peak intensities (Fig.1C). Moreover, PMF
241 profiles of controls, T1+T2 patients and T3+T4 patients were further analyzed by a
242 multivariate statistical analysis including 2D-cluster plot analysis and principal component
243 analysis (PCA) by the *ClinProTools 2.0* software. 2D-cluster plot analysis demonstrated
244 represents the best separating peaks in two dimensional spaces (Fig. 2A), while 3-D view of
245 PCA scores plot analysis indicated a well differential distribution of mass peaks among
246 controls, T1+T2 patients and T3+T4 patients (Fig.2B). In addition, three peaks with
247 significantly different intensity were showed in average spectra profiles from controls, T1+T2
248 patients and T3+T4 patients (Supplemental Table 2). These three peaks had a mass of 2919
249 Da, 5592 Da, and 4373 Da, respectively, being potential OSCC biomarkers identified by
250 functionalized C8-bead purification, MALDI TOF MS and the *ClinProTools 2.0* software. Of
251 threes three OSCC biomarkers, occurrence frequency and peak intensity of 2919 Da, but not

252 5592 Da, and 4373 Da, was higher in saliva from OSCC patients than in those from controls
253 (Fig. 1C). Subsequently, the peptide mass of 2919 Da was chosen for amino acid sequencing
254 by MALDI-TOF MS/MS, and then the sequence of the peptide mass of 2919 Da was
255 CNSWEVNLQSISEFIINNRNYSTK (Fig. 3), which was identified to match the residues
256 176-199 of zinc finger protein 510 (ZNF510). We suggested the 24-mer NZF510 peptide as a
257 potential saliva biomarker for diagnosing OSCC.
258
259 *3.2. Validation of 24-mer ZNF510 peptides in OSCC saliva*
260 To analyze the level of the ZNF510 peptide in saliva using ELISA, the ZNF510 peptide
261 was synthesized using stepwise solid phase peptide synthesis procedures, and then was used
262 for the immunization of rabbits. Endpoint titer of antisera after five immunizations was
263 greater than 1:10000 using a direct ELISA assay with ZNF510 peptide-coated microtiter
264 plates (Supplemental Fig. 1A). In addition, minimum detectable level of ZNF510 peptide in
265 direct ELISA was lower than 0.25 ng of ZNF510 peptide using rabbit antisera at a 1:1000
266 dilution (Supplemental Fig. 1B). The results demonstrated the binding specificity of rabbit
267 anti-ZNF510 peptide sera which could be used for detection of ZNF510 peptide in salivary
268 samples. Competitive inhibition ELISA indicated that ZNF510 peptide at a dose of 25 μg
269 showed 1.7%, 21.8%, and 46.9% inhibition of the binding of anti-ZNF510 peptide sera with
270 control saliva, T1+T2 saliva and T3+T4 saliva, respectively (p<0.01) (Fig. 4A). ZNF510

peptide existed in OSCC saliva, detectable by ELISA with rabbit anti-ZNF510 peptide sera. Direct binding ELISA with 1:1000 dilution of anti-ZNF510 peptide sera showed the level of ZNF510 peptide in T3+T4 saliva (OD405 of 0.372) and in T1+T2 saliva (OD405 of 0.368) as significantly higher than control saliva (OD405 of 0.208) ($p<0.01$) (Fig. 4B). Competitive inhibition ELISA and direct binding ELISA correlated anti-ZNF510 peptide levels in OSCC patients with tumor size, as represented by the UICC TNM staging system.

3.3. Analysis of ZNF510 proteins expression in OSCC tissues, saliva and cell lines

We further validated ZNF510 protein level in OSCC and OSCC-free control tissues using immunohistochemical staining with rabbit anti-ZNF510 peptide sera. As shown in Fig. 5**,** some expression of NZF510 was observed in nuclei of OSCC-free control tissues, but not evident in the vascular endothelium region. However, strong expression of ZNF510 appeared in OSCC cytoplasm, particularly in interstitial tissue as well as in vascular endothelium especially near the basal layer in OSCC tissues. In addition, Western blotting analysis with rabbit anti-ZNF510 peptide sera showed a 72-kDa immunoreactive band as ZNF510 protein (683 amino acid residues) in OSCC saliva and lysates of human OSCC cell line HSC3, but OSCC-free saliva and non-OSCC cell lines (data not shown). Immunohistochemical comparison of OSCC and OSCC-free tissues and Western blotting analysis of OSCC and OSCC-free saliva and cell lines confirmed the occurrence of ZNF510 peptide increase in

OSCC saliva as a potential biomarker for OSCC progression from early to late carcinomas.

3.4. Accuracy of salivary ZNF510 peptides for prediction of early OSCC stages

We also analyzed receiver operating characteristic (ROC) curves to confirm ability of ZNF510 to distinguish OSCC patients from healthy individuals. Area under the ROC curves (AUC) predicted sensitivity and specificity of ZNF510 peptide-based ELISA for detection of staging in patients with OSCC. AUC for saliva ZNF510 peptide of OSCC Stages T1+T2 and healthy individuals was 0.95 (with 95% CI for area being between 0.40 and 0.82) (Fig. 6A); that of OSCC Stages T3+T4 and healthy individuals was 0.88 (with 95% CI for area between 0.27 and 0.66) (Fig. 6B). These indicate ZNF510 peptide-based ELISA had significant accuracy in detection of early stages from patients with OSCC.

4. Discussion

This study contrasted peptide profiles of saliva from OSCC patients with an OSCC-free control group, using C8/MALDI-TOF MS analysis and *ClinProTools* software. Our results indicate peptide mass of 2919 Da as significantly elevated in saliva of OSCC patients (Figs. 1-2, Supplemental Table 2), which was sequenced and matched with the residues 176-199 of ZNF510 (Fig. 3). Subsequently, we specifically focused on 24-mer ZNF510 peptide as a

potential saliva biomarker for OSCC progression. Significant increase in level of salivary ZNF510 peptides in OSCC patients correlated with increasing tumor size, as plotted by the UICC TNM staging system (P<0.01) (Figs. 4). Immunohistochemistry stain indicated strong ZNF510 expression in cytoplasm of OSCC versus control cells (Fig. 5). Moreover, AUROC of a salivary ZNF510 peptide-based ELISA for diagnosis of early-stage oral cancer revealed that salivary ZNF510 peptide serves as an early-stage biomarker for oral cancer (Fig. 6). Our study utilized saliva proteomics to ferret out potential OSCC biomarkers, targeting ZNF510 levels in saliva at different stages.

ZNF proteins are family members containing small DNA recognition motifs, comprising about 30 amino acid residues and a zinc ion [23]. Functions of ZNF proteins were thought involved in controlling cell growth, proliferation, differentiation, and apoptosis [24]. Prior study reported ZNF652 as able to promote tumorigenesis by regulation of CBFA2T3 protein [25]. ZNF652 protein was detected at lower levels in vulvar carcinoma cells [26], suggesting its possible role in regulating cell proliferation. Another such protein is ZNF28, found to correlate with pathogenesis of melanoma [27]. ZNF410 is reportedly associated with human esophageal squamous cell carcinoma [28]. All these portend expression level of specific ZNF as a biomarker candidate in various types of tumors. Our study first demonstrated salivary level of ZNF510 as strongly correlated with OSCC stages. Since ROC analysis indicated salivary ZNF510's predictive value for OSCC progression: salivary ZNF510 expression level

in Stages T3+T4 was more abundant than in Stages T1+T2 and hence holds promise for detection of OSCC, especially as a biomarker of early disease.

ZNF510 peptide-based ELISA assay showed mean level of salivary ZNF510 peptide in OSCC patients as 12.7-fold higher in T1+T2 group and 27.6-fold in T3+T4 group compared with controls (Fig. 4A). Overall specificity and sensitivity of salivary ZNF510 peptide-based ELISA were higher than 95% in OSCC patients (Figs. 4B and 6). By contrast, sensitivity of salivary solCD44 levels for detecting HNSCC patients ranged from 62% to 70%, specificity from 75% to 88% [16]. The sensitivity and specificity values of CycD1 and Maspin as candidate OSCC markers were 100% [17]. In addition, combination of M2BP, MRP14, CD59, profilin, and catalase as candidate biomarkers yielded a receiver operating characteristic value of 93%, sensitivity of 90%, and specificity of 83% in detecting OSCC [18,19]. Comparison of salivary ZNF510 peptide with these reported candidate markers indicated that the receiver operating characteristic value, specificity and sensitivity salivary transferrin were similar to those of reported candidate OSCC markers. Of candidate markers, only salivary ZNF510 peptide proved able to detect early-stage oral cancer. In the OSCC-free control group, four samples exhibiting elevated levels of salivary ZNF510 peptide were from individuals suffering from right parotid mixed tumor, alcoholic liver cirrhosis, nasal polyposis or bronchial asthma. Combination of salivary NZF510 peptide with other OSCC markers could rule thus out false positive and confirm early diagnosis of OSCC.

346 Results suggested peptide mass of 2919 Da in salivary peptide profiles and levels of
347 ZNF510 peptide in saliva as correlated with OSCC progression; molecular mechanism of
348 ZNF510 in tumorigenesis and progression OSCC needs further demonstration. Also, due to
349 heterogeneous properties of OSCC progression, a panel set of several potential biomarkers
350 may make diagnosis more precise than any marker alone. However, developing noninvasive
351 diagnosis with salivary biomarkers as means of early diagnosis and therapeutic target of
352 cancers is important in clinical research.

353

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357

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432

433 # Figure legends

434

435 **Figure 1. Peptide profiles of salivary samples from control individuals and OSCC**
436 **patients using C8 bead/ MALDI-TOF MS and *ClinProt* software. S**alivary samples from
437 normal individuals (A) and OSCC patients (B) were fractionated by use of C8 beads, then
438 subjected to MALDI-TOF MS and analyzed with *ClinProt* software. Pseudo-gel views (C) of
439 peptide profiles of salivary samples from control individuals (lower column), T1+T2 OSCC
440 patients (middle column) and T3+T4 OSCC patients (upper column) were shown with with
441 the calculated molecular weight (m/z values) along the x-axis and relative intensity along the
442 y axis using *ClinProt* software.

443

444 **Figure 2. Two-dimensional -cluster plot analysis (A) and three-dimensional view**
445 **displays (B) of the principal component analysis of peptide profiles using *ClinProt***
446 **software.** Red spots represent control individuals; green spots represent T1+T2 patients; blue
447 spots represent T3+T4 patients.

448

449 **Figure 3. Identification of mass peak 2919 Da from saliva of OSCC patients using**
450 **MALDI-TOF/TOF MS analysis.** Amino acid sequence
451 CNSWEVNLQSISEFIINNRNYSTK was determined from mass differences in y- and

452 b-fragment ions series and matched residues 176–199 of ZNF510.

453

454 **Figure 4. Correlation of salivary ZNF510 peptide levels with OSCC staging**. Salivary
455 ZNF510 peptide levels in control individuals and OSCC patients were detected using
456 competitive binding inhibition ELISA (A) and direct binding ELISA (B). The 1000-fold
457 dilution sera were incubated with or without 25 μg of ZNF510 peptide, then the mixtures
458 were added into NF510 peptide-coated microtiter plates for competitive binding inhibition
459 ELISA. In addition, the 1000-fold dilution sera were directly added into NF510
460 peptide-coated microtiter plates for direct binding ELISA.

461

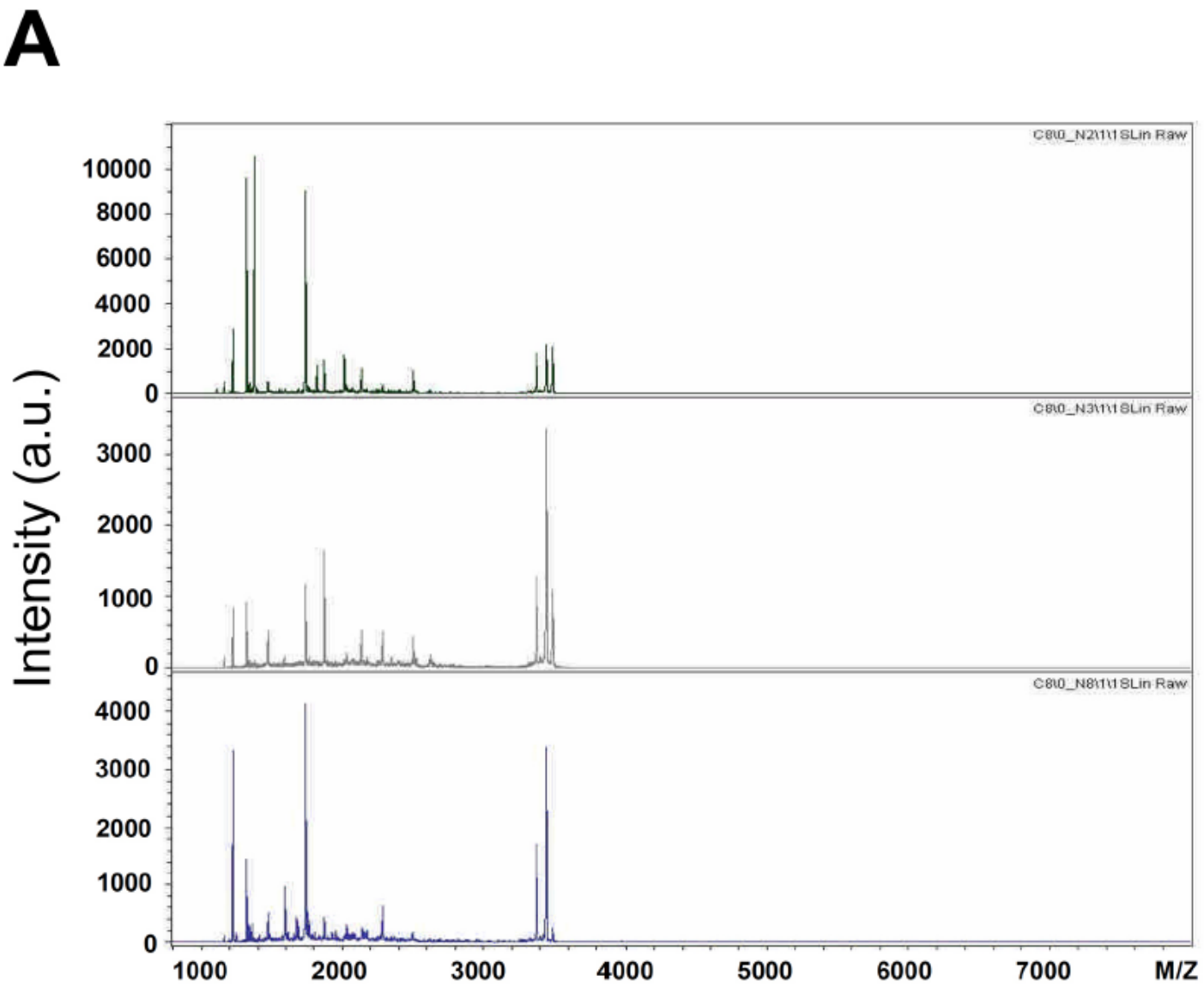
462 **Figure 5. Immunohistochemical analysis of ZNF510 expression in OSCC.** Tissue sections
463 from healthy control subjects (A, B, C, D) and OSCC patients with T2 stages (E, F, G, H) was
464 analyzed using immunohistochemical staining with rabbit anti-ZNF510 sera.
465 Immunoreactivity of NZF510 was developed by HRP-conjugated anti-rabbit IgG antibodies
466 and 3,3'-diaminobenzidine as substrate. The figures were photographed under ×100 (A, C, E
467 and G) and ×400 (B, D, F and H) magnification.

468

469 **Figure 6. Receiver-operating characteristic curves of salivary ZNF510 peptide–based**
470 **ELISA for the prediction of oral cancer of different stages T1+T2 (A) and T3 + T4(B).**

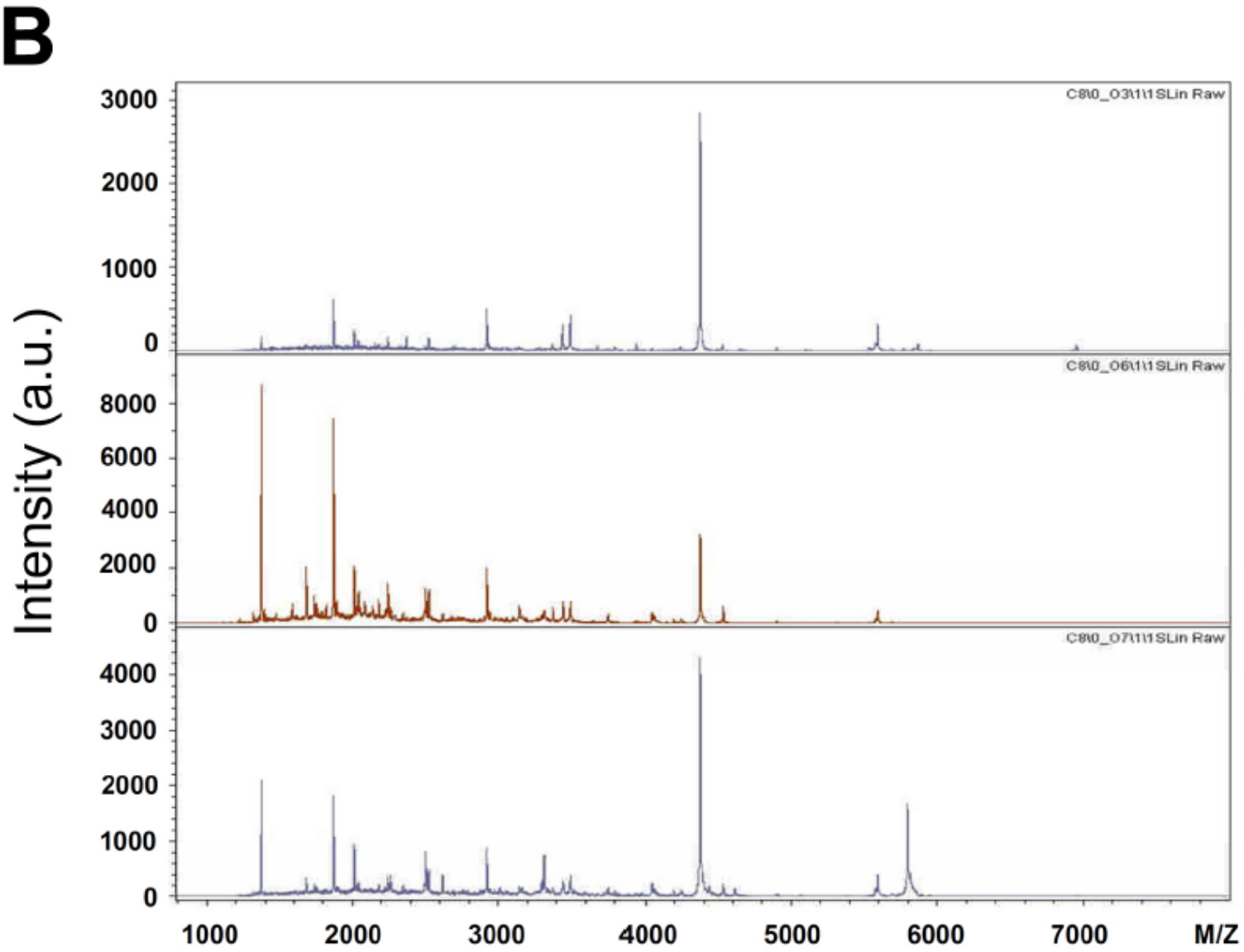
Figure(s)

Fig. 1



Figure(s)

Fig. 1



Figure(s)

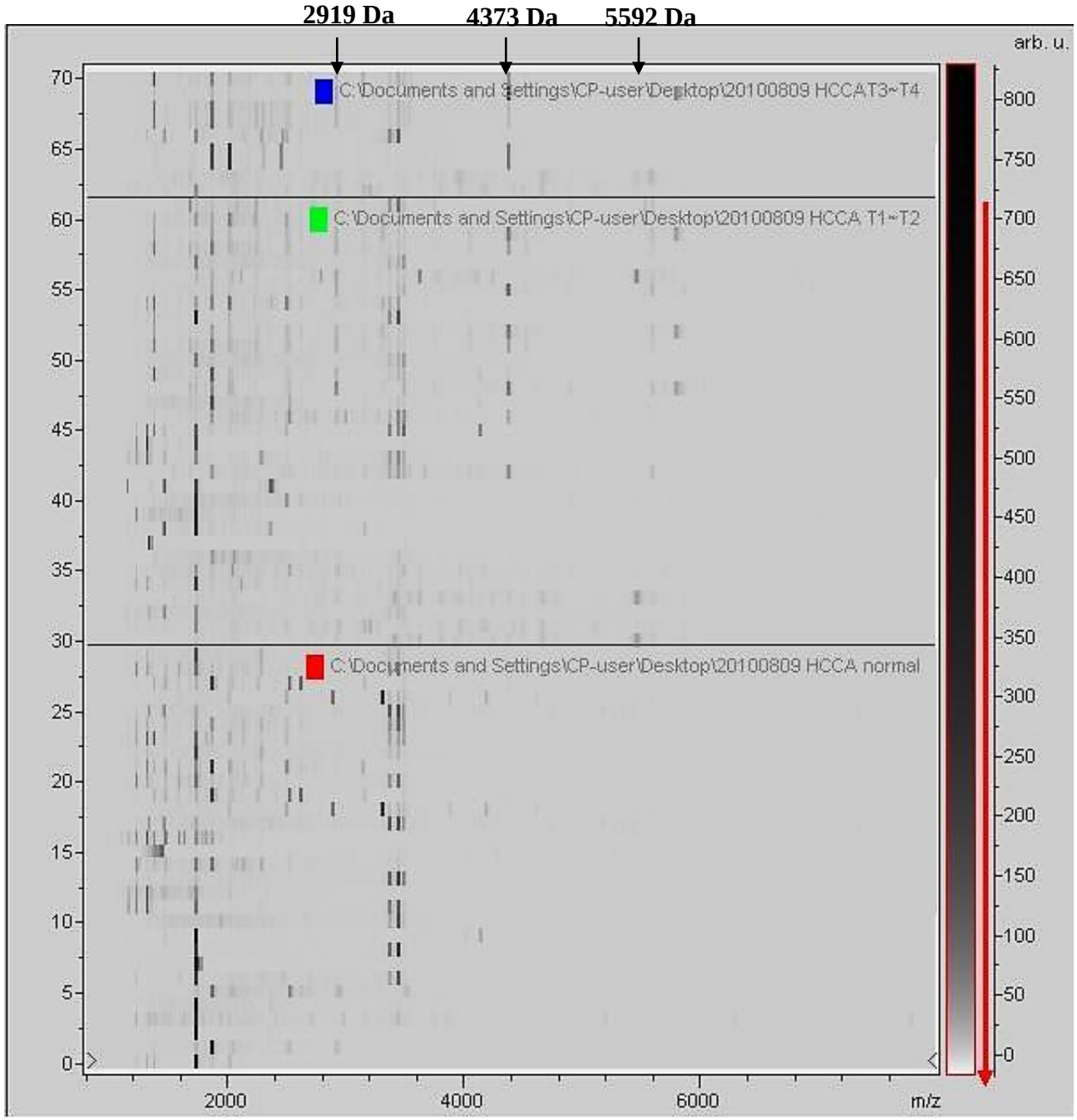
Fig. 1

C

OSCC patients with T3+T4 stages (n=9)

OSCC patients with T1+T2 stages (n=32)

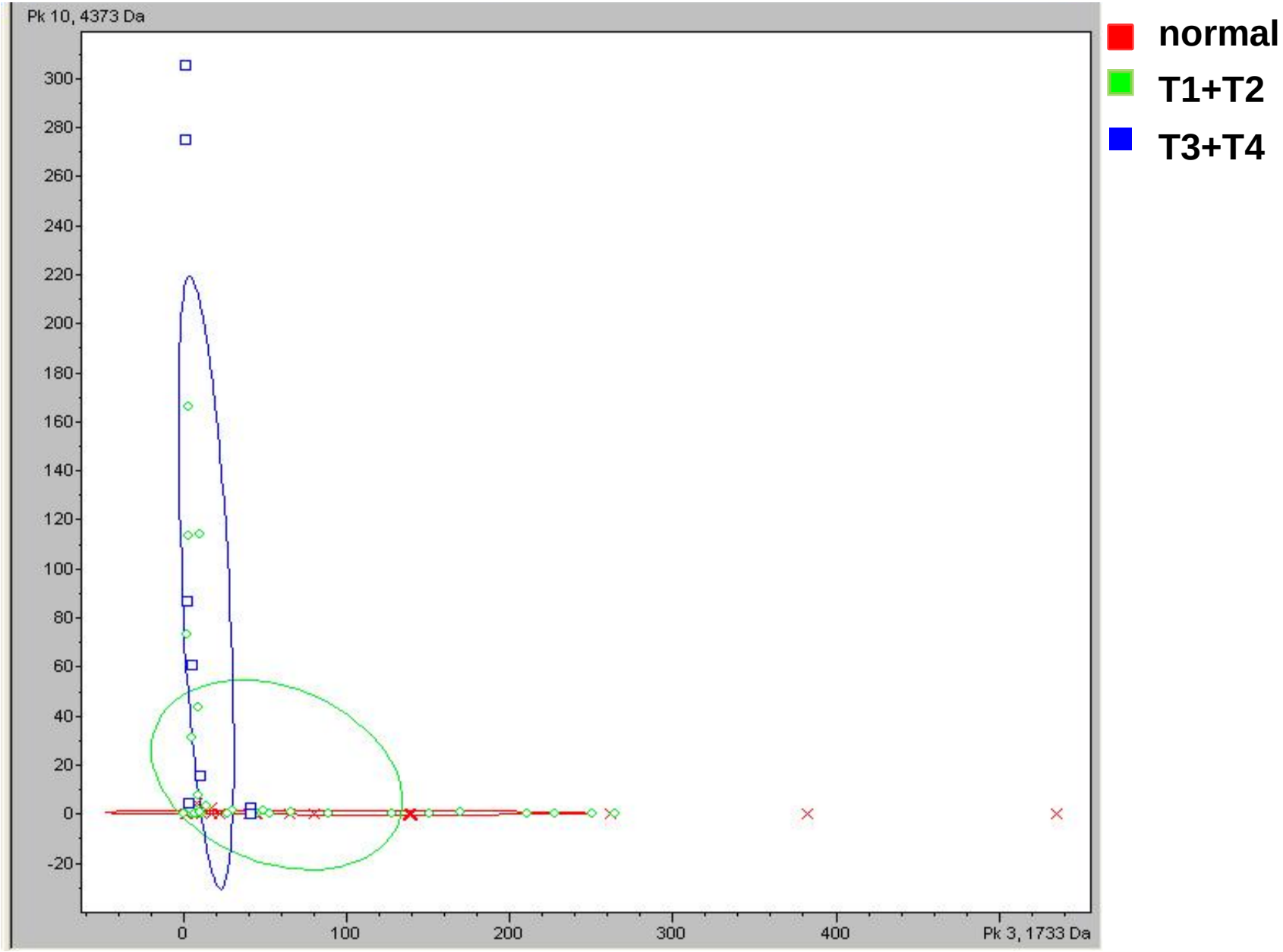
Control subjects (n=30)



Figure(s)

Fig. 2

A



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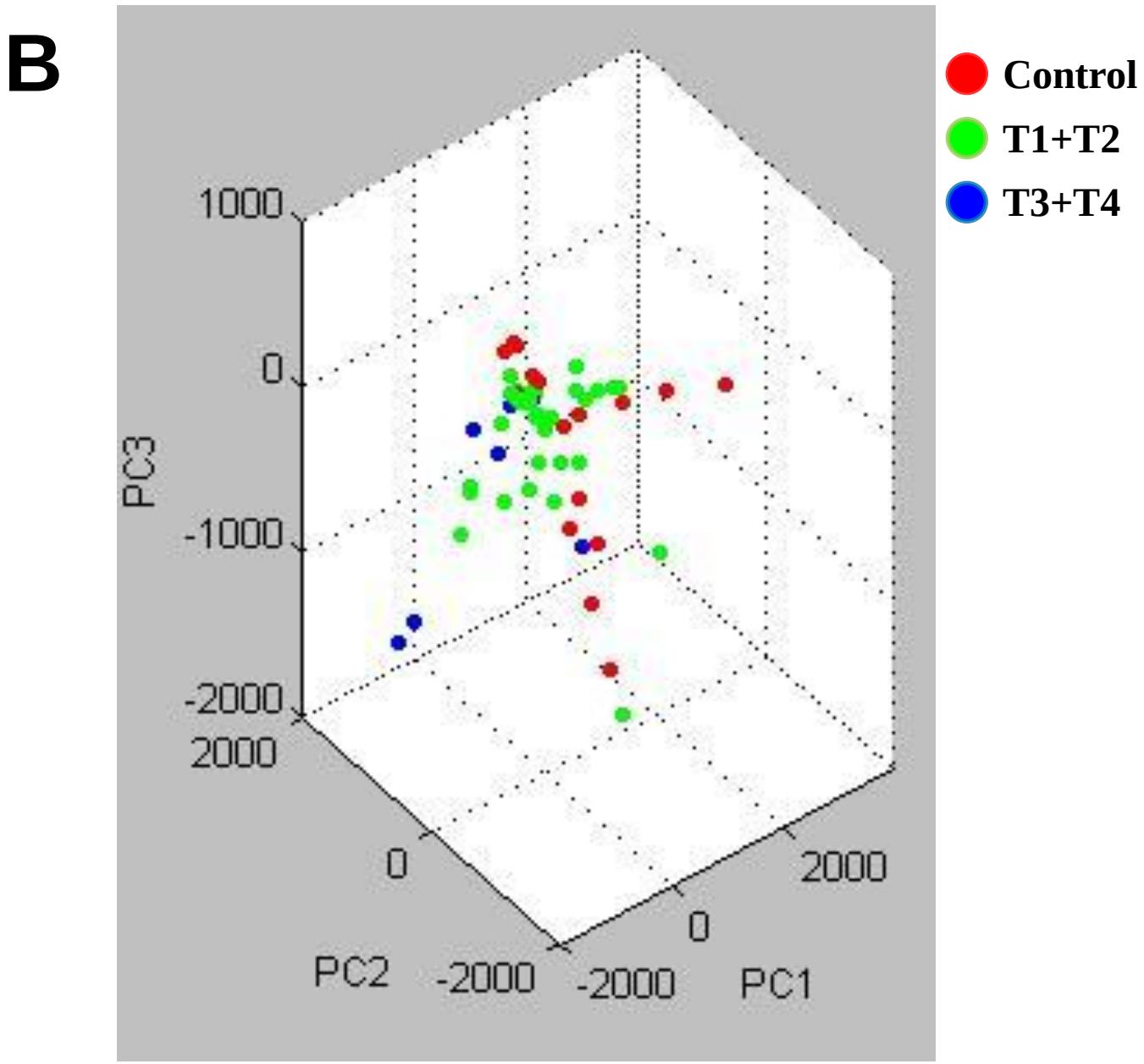

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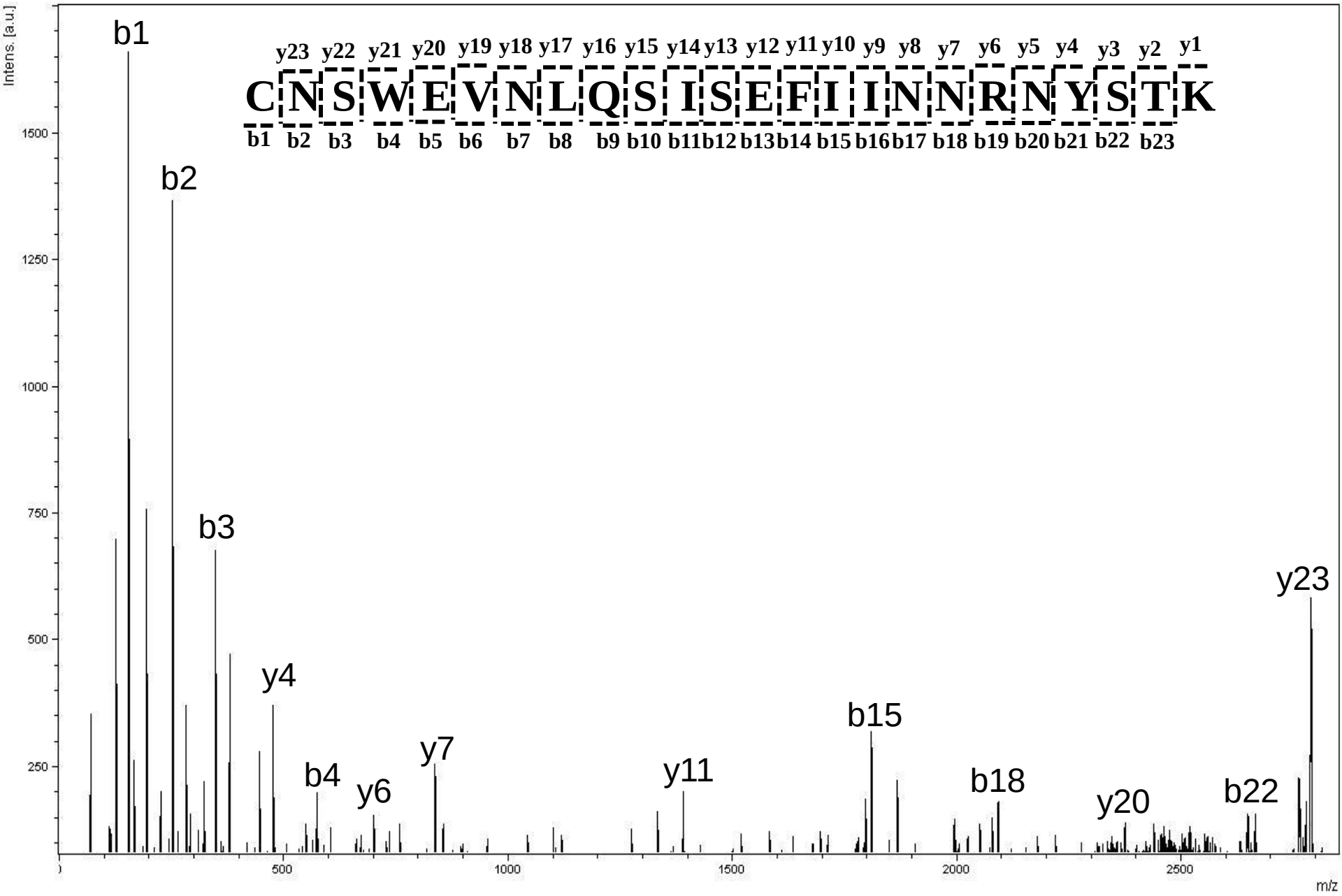

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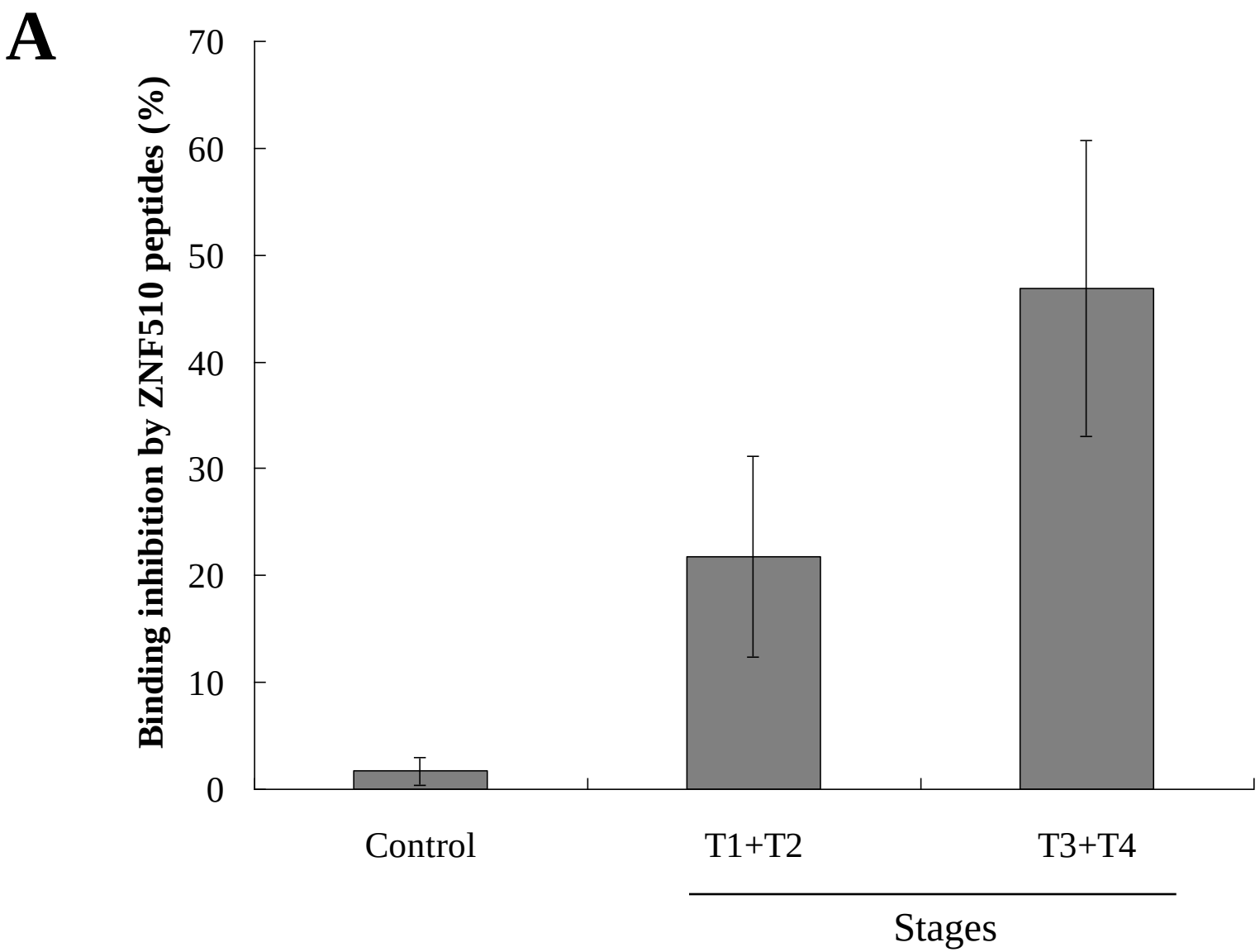

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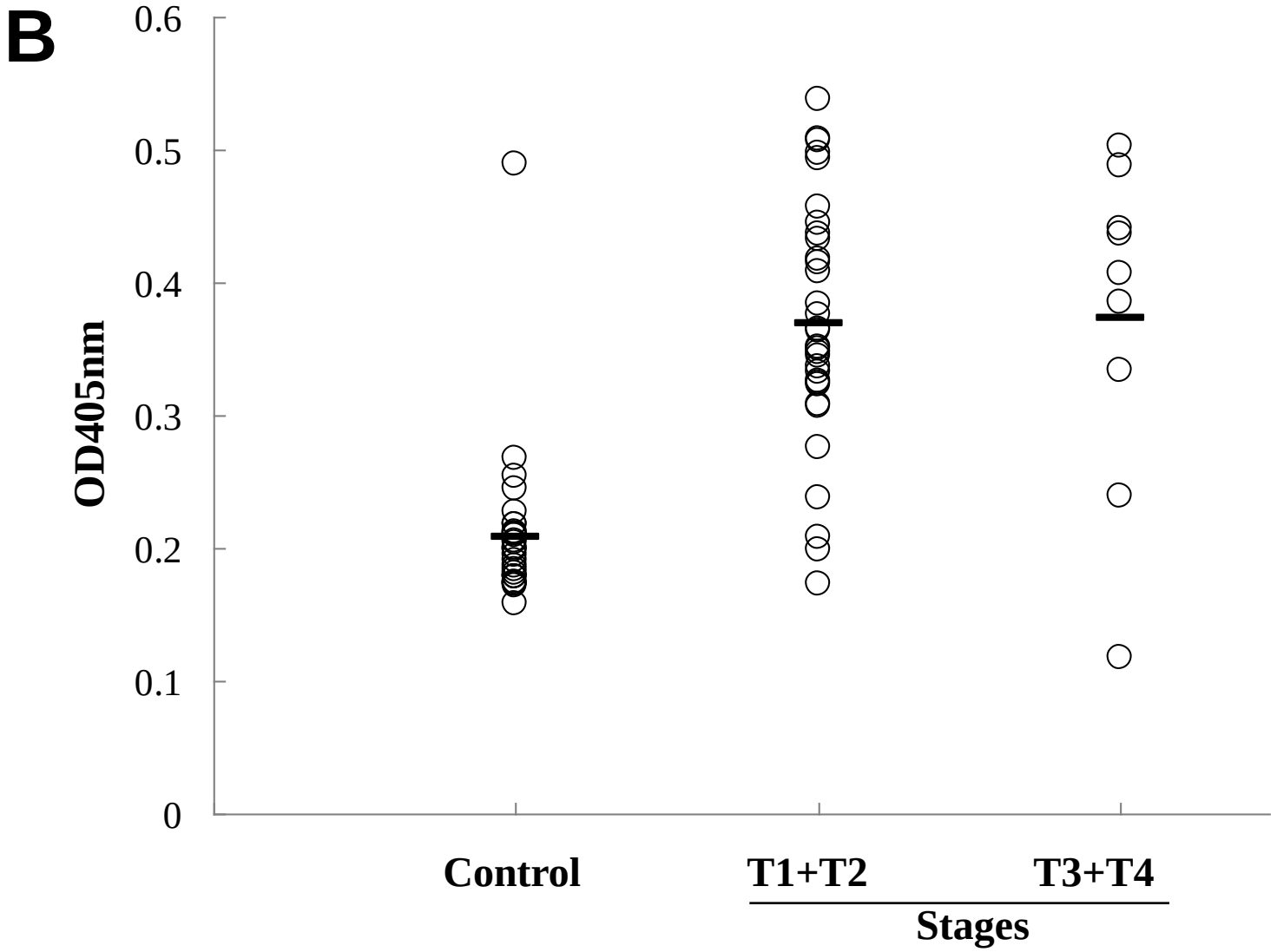
Fig. 4



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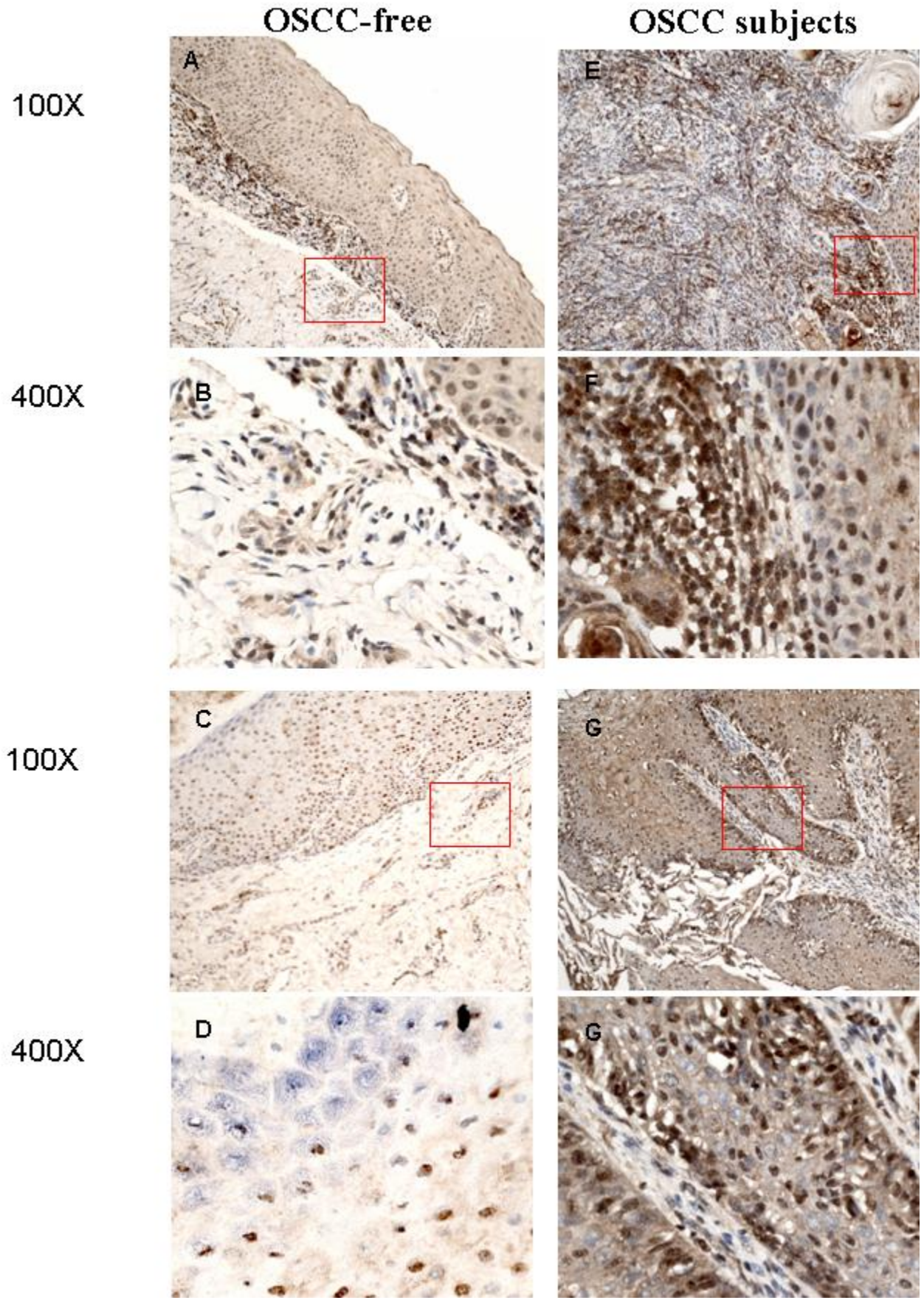

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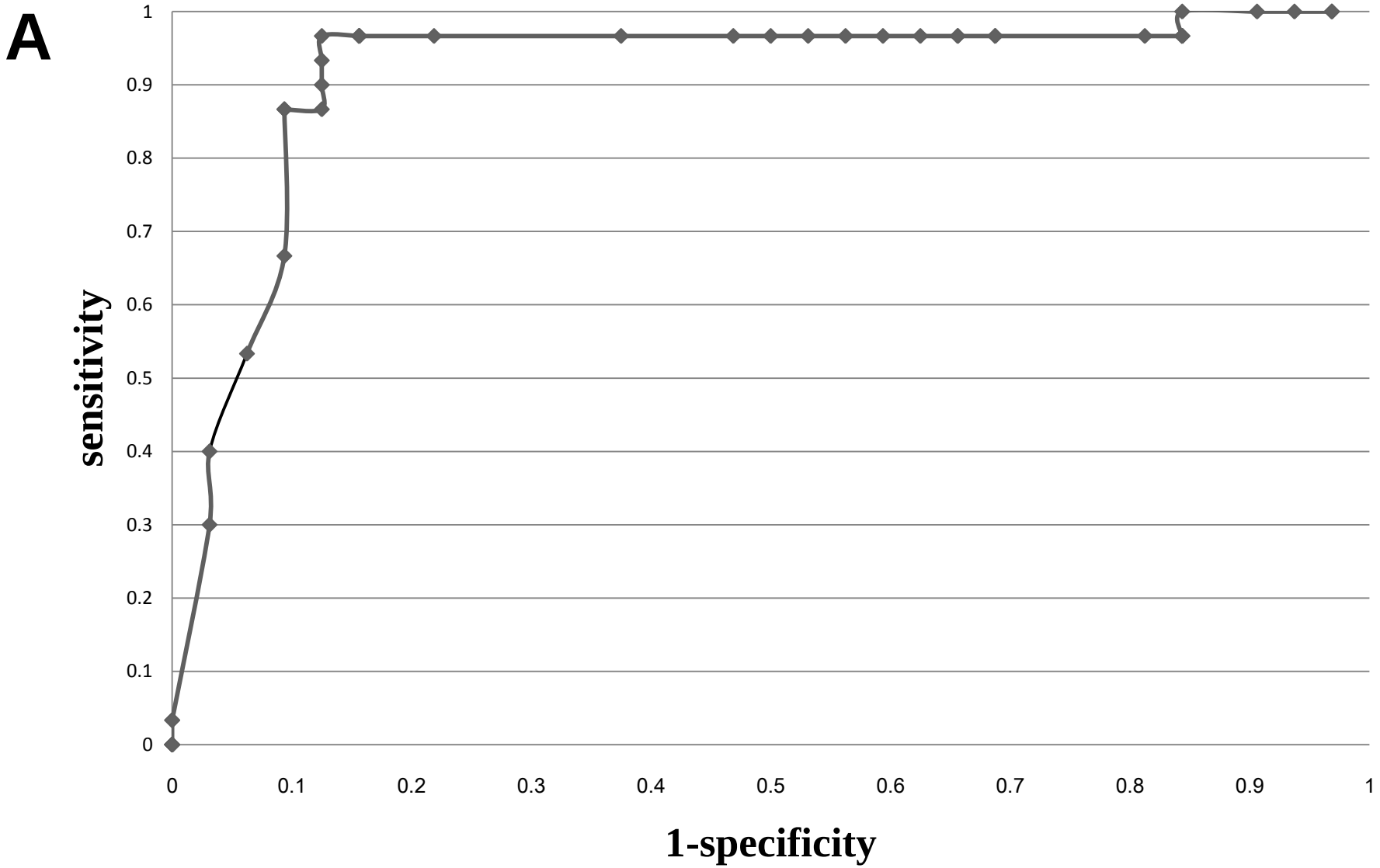

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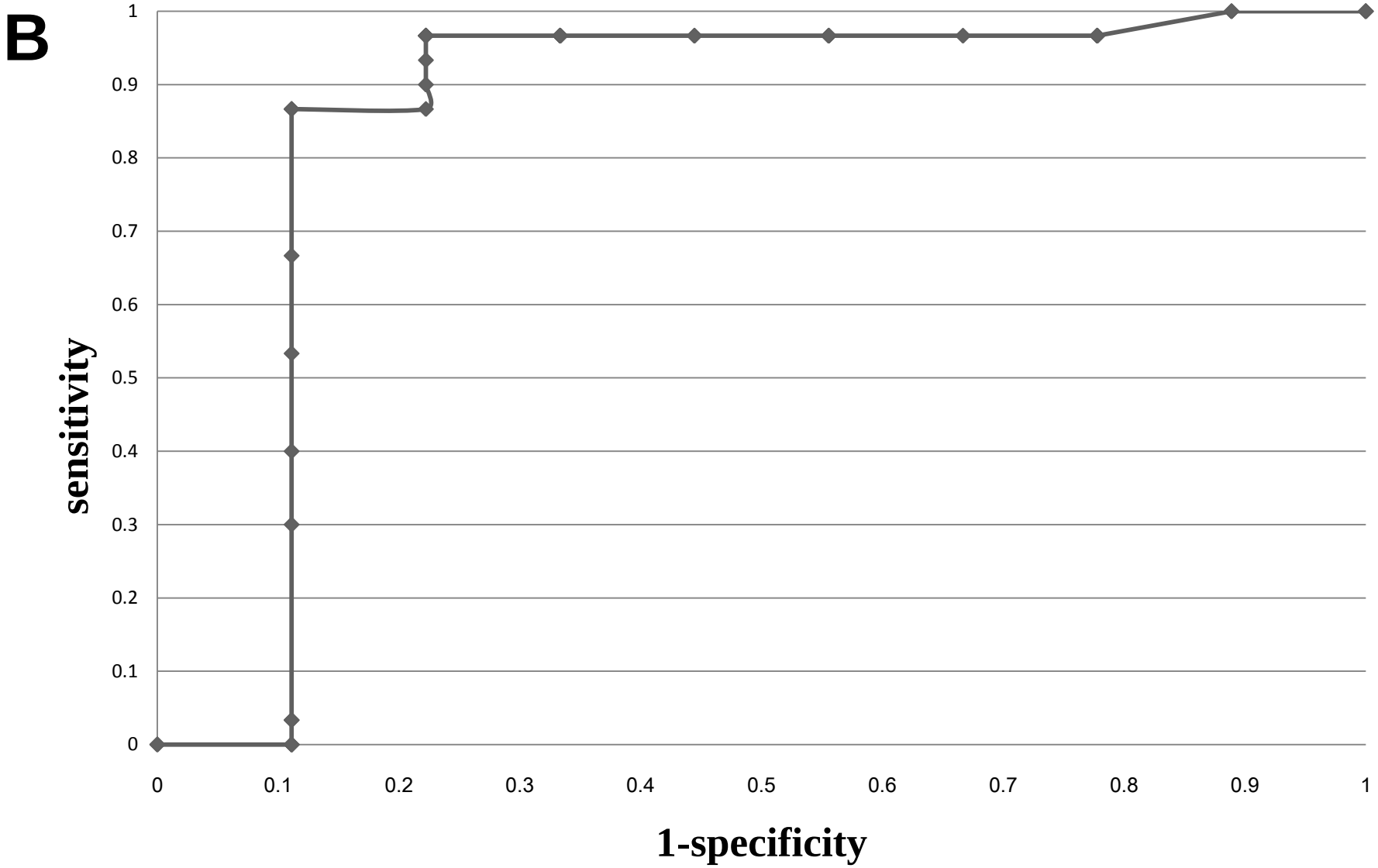
Fig. 6



Supplementary Material

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Supplementary Material

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