Overexpression of Interleukin-8 and Interleukin-13 as potent immune markers associated with survival and dietary habits in esophageal squamous cell carcinoma.

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Abstract

Background Interleukin-8 (IL8), Interleukin-12 (IL12) and Interleukin-13 (IL13) are cytokines that play regulatory role in cancer pathogenesis. We analysed their expression profile to evaluate as molecular biomarkers of esophageal squamous cell carcinoma (ESCC) and their association with different parameters.

Methods Expression analysis of IL8, IL12 and IL13 were performed by Real time qPCR in blood and tumor tissue of 120 ESCC patients. The expression profiles were associated with different clinicopathological and dietary factors. Survival and hazard analysis were also performed.

Results When compared to normal controls, IL8 expression showed upregulation in 83% tissue samples (p=0.000) and 62% blood samples (p=0.388), IL12 expression showed upregulation in 62% tissue samples (p=0.435) and 57% blood samples (p=0.222) and IL13 expression showed upregulation in 83% tissue samples (p=0.001) and 68% blood samples (p=0.312). Significant positive correlation ($p<0.05$) was observed between tissue and blood level expression of IL8, IL12 and IL13. Different clinicopathological factors and dietary habits showed significant association ($p<0.05$) with IL8, IL12 and IL13 expression.. Statistically significant positive correlation were observed for IL8 and IL13 expression in tissue as well as IL13 and IL12 expression in both tissue and blood. Also significant negative correlation of IL8 and IL12 expression in blood and tissue were also observed. Tumor stage, node stage, metastasis, consumption of betel nut, tobacco, alcohol, hot food, smoked food, spices, IL8 expression in blood, IL13 expression in tissue and IL12 expression in blood and tissue showed significant association ($p<0.05$) with survival of ESCC patients.

Conclusions Altered expression of IL8, IL12 and IL13 may be associated with ESCC progression. Overexpression of IL8 and IL13 in tissue samples may be potential biomarkers for ECSS screening. Additionally, results from both survival and hazard analysis data indicate the effects of various parameters on the survival and mortality rate of ESCC patients.

Keywords

Interleukin-8, Interleukin-12, Interleukin-13, Esophageal squamous cell carcinoma, survival analysis, dietary habits.

Introduction

Esophageal cancer represents the seventh most common cancer worldwide and it is the sixth most common reason of cancer-related death worldwide with a survival rate of only 15-20 % in five years [1, 2]. Esophageal squamous cell carcinoma (ESCC) is reported as the most prevalent histological form of esophageal cancer in the world [3]. Patients with ESCC often present at an advanced stage when diagnosed because there are ineffective early detection tools, which has a negative impact on the patients' prognosis. Detection of molecular biomarkers might open up new, efficient means for tumour diagnosis, screening, monitoring, and prognosis [4, 5].

Tumor microenvironment consists of many different types of cells, which produces different kind of cytokines that can either enhance or inhibit cancer cell proliferation. These all interact with one another to play their role in tumor pathogenesis [6, 7]. Interleukin-8 (IL8) is a pro-inflammatory cytokine produced by different cell types in response to tissue infection, inflammation or injury [8-10]. It is also associated with the development of different types of cancer like lung cancer, prostate cancer, breast cancer, colorectal cancer, etc. [8, 11]. It plays

a dual potential role in tumor microenvironment by directly promoting tumor survival and indirectly facilitating tumor progression by affecting components of tumor microenvironment, which include epithelial-to-mesenchymal transition, pro-angiogenesis process, tumor cell proliferation and inhibition of anti-tumor immunity [8]. Interleukin-12 (IL12), an essential pro-inflammatory heterodimeric cytokine, is primarily produced by antigen presenting cells in response to infection. It plays an important role in connecting adaptive and innate arm of immune systems and stimulates the activity of natural killer cell and T cell and induces production of interferon gamma [12, 13]. It is a potent agent in enhancing antitumor immune responses and plays important roles in the regulation of cellular immunity [14]. It has been considered as an essential immunotherapeutic agent for combinatorial cancer treatments [13]. Interleukin 13 (IL-13) is an immunoregulatory cytokine, synthesized primarily by activated T-helper 2 cells, but also by B cells, natural killer, dendritic cells, mastocytes, basophils, etc. [15, 16]. IL13 and its receptors play an essential role in the proliferation of cancer cells and other biological behaviours like invasion, migration etc. and enhance the malignant phenotype. In many human cancers, the presence of IL13 and its receptors are reported to have association with chemosensitivity, apotosis and cancer prognosis [17-19]. In this study we have analysed the expression of cytokine IL8, IL12 and IL13 between blood and tissue samples and also among male and female patients of ESCC. We further evaluated the association between the expression profile of these cytokines and different clinicopathological and dietary factors in ESCC patients.

The survival rate of esophageal cancer is quite low because most cases of this cancer are detected at a late stage. Several factors affect the survival of esophageal cancer patients and these factors may vary in esophageal cancer patients from different populations. Therefore, research that identifies esophageal cancer risk factors is necessary to enhance patient

prognosis and survival rates [20]. In this study, survival and hazard analysis were performed to check the impact of different risk factors on survival or mortality rate of ESCC patients.

Materials and Methods

Sample collection

Blood samples were collected using standard venipuncture and tumor tissues as well as adjacent normal tissues were collected by biopsy from 120 ESCC patients with written informed consent. The diagnosis of ESCC was confirmed by upper gastrointestinal endoscopy (UGI endoscopy) and by pathological analysis of tumor biopsies. An equal number of healthy individuals (age and sex matched) were also enrolled in the study. The study was approved by the Institutional Ethical Committee of the Gauhati Medical College and Hospital, Guwahati (MC/217/2016/Pt-I/20; dated 22 December 2016) and North East Cancer Hospital, Jorabat (IEC/2018/06/NP/12; dated 27 August 2018) and all the procedures were in accordance with the Helsinki Declarations and with the ethical standards of the Institutional Ethical Committee. Histopathology grade, stages of tumor, node and metastasis were categorized according to AJCC (American Joint Committee on Cancer) manual of cancer staging. Dysphagia grade was determined using the modified O'Rourke grading system. Amount of tea consumption was categorized into low (who consumed1-2 times/day), medium (who consumed 3-4times/day) and high amount (who consumed 5 or more times/day). Amount of khar consumption was categorized into no (who don't consume), low (1-2 times/month), medium (3-4 times/month) and high (5 or more times/month) amount.

mRNA expression profile analysis by Real Time PCR

RNA isolation was done manually using the Trizol reagent from homogenized tissue and blood samples according to the manufacturer's instruction. Reverse transcription was done to prepare complementary DNA (cDNA) using iScript™ Reverse Transcription Reagents (Bio-Rad Laboratories, Inc.). Real time qPCR was performed using SYBR green master mix in a Rotor-Gene Q (Qiagen) for analysis of mRNA expression. Human housekeeping gene β-actin was considered as standard reference for normalization. The level of expression of the targeted genes was calculated using the formula Comparative Ct ($2^{-\Delta\Delta Ct}$) method. If the calculated level of expression was <1, the targeted gene was downregulated and if the level of expression was >1, the targeted gene was upregulated. The primer used for IL8, IL13, IL12 and β-actin genes were: Forward (F): 5′-TCTGTCTGGACCCCAAGGAA-3′, Reverse (R): 5-GCAACCCTACAACAGACCCA-3′; F: 5′-GCACAGACCAAGGCAAATG-3′, R: 5′-GCAGAATGAGTGCTGTGGA-3′; F: 5′-TGATGAAGAAGCTGCTGGT-3′, R: 5′-GTCAGAGGGGACAACA-3′ and F: 5′-AGATGTGGATCAGCAAGCAG-3′, R: 5′-GCGCAAGTTAGGTTTTGTCA-3′ respectively.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 18.0 was used for all statistical analysis. All the data were expressed as mean ± standard deviation and two tailed tests were taken into consideration. All the tests were considered statistically significant with a p-value < 0.05. Student's paired t test was used to compare mRNA expression levels in cases and controls. The parametric independent sample's t test and one way ANOVA or the non-parametric Mann-Whitney U test and Kruskal Wallis H test were performed to study the association with different parameters. Kaplan–Meier method of survival analysis was done using the log-rank test and univariate model of Cox's regression was used to detect hazard outcomes for different risk factors.

Results

IL8, IL12 and IL13 expression in blood and tissue samples of ESCC patients

IL8 expression was upregulated in 83% ESCC cases with a mean fold change of 2.63±1.06 and downregulated (0.40±0.24) in 17% ESCC cases in tissue samples (p=0.000). In blood samples, IL8 expression was upregulated (2.37±1.19) in 62% cases and downregulated (0.28±0.22) in 38% cases (p=0.388). While analyzing IL12 expression, 62% cases showed upregulation (1.77±0.75) and 38% cases showed downregulation (0.46±0.31) in tissue samples (p=0.435). In blood samples, 57% cases showed upregulation (2.10±0.75) and 43% cases showed downregulation (0.42±0.30) of IL12 (p=0.227). Expression of IL13 was upregulated (2.27±0.91) in 83% cases and downregulated (0.46±0.26) in 17% cases in tissue samples (p=0.001). While analyzing blood samples, 68% cases showed upregulation (2.30±1.08) and 32% cases showed downregulation (0.49±0.30) of IL13 (p=0.312). Tissue and blood level expression of IL8, IL12 and IL13 in ESCC patients are represented in Table 1. Box plot representation of IL8, IL12 and IL13 expression in ESCC patients are represented in Fig 1 and box plot representation of IL8, IL12 and IL13 expression in ESCC patients compared to normal control are represented in Fig 2.

Fig 1. Box plot representation of IL8, IL12 and IL13 expression in tissue and blood level of ESCC patients.

(A) IL8 expression in tissue; (B) IL8 expression in blood; (C) IL13 expression in tissue; (D) IL13 expression in blood; (E) IL12 expression in tissue; (F) IL12 expression in blood. [Note: Box plot explanation: Dark horizontal bar within box–median; upper horizontal line of box-

75th percentile; lower horizontal line of box-25th percentile; whiskers and dots-range in the box plot.]

Fig 2. Box plot representation of IL8, IL12 and IL13 expression in tissue and blood samples of ESCC patients compared to normal control.

(A) Relative IL8 expression in tumor tissues and adjacent normal tissues; (B) Relative IL8 expression in blood samples and control samples; (C) Relative IL13 expression in tumor tissues and adjacent normal tissues; (D) Relative IL13 expression in blood samples and control samples; (E) Relative IL12 expression in tumor tissues and adjacent normal tissues; (F) Relative IL12 expression in blood samples and control samples. [Note: Box plot explanation: Dark horizontal bar within box–median; upper horizontal line of box-75th percentile; lower horizontal line of box-25th percentile; whiskers and dots-range in the box plot.]

Table 1. Expression profile of IL8, IL13 and IL12 gene in both blood and tissue of ESCC patients.

Gene	Upregulation	Downregulation	p value (Case versus control comparison)
IL8 tissue	2.63±1.06 (N=100)	0.40±0.24 (N=20)	0.000*
IL8 blood	2.37±1.19 (N=74)	0.28±0.22 (N=46)	0.388
IL12 tissue	1.77±0.75 (N=74)	0.46±0.31 (N=46)	0.435
IL12 blood	2.10±0.75 (N=68)	0.42±0.30 (N=52)	0.227
IL13 tissue	2.27±0.91	0.46±0.26	0.001*

	(N=100)	(N=20)	
IL13 blood	2.30±1.08 (N=81)	0.49±0.30 (N=39)	0.312

Note: Data are represented as mean ± standard deviation. * Statistically significant p value. p value was calculated using the relative mRNA expression in tissue and blood samples of ESCC patients compared to normal control. [Abbreviation: N= Number of patients.]

Association of IL8, IL12 and IL13 expression with different clinicopathological and dietary factors in ESCC

While targeting the IL8 expression, significant associations ($p<0.05$) were observed in age group, dysphagia grade, metastasis, consumption of alcohol, betel nut, spices, meat and hot food in tissue samples of male cases (S1 Fig), while age group, dysphagia grade, tumor stage, consumption of betel nut, hot food, smoked food, spices and amount of tea consumed showed significant differences in female cases (S2 Fig). For blood samples, male patients showed significant association with age group, dysphagia grade, consumption of tobacco, betel nut, hot food, spices and type of tea consumed (S3 Fig), whereas female patients showed an association with age group, dysphagia grade, tumor stage, consumption of betel nut, spices and hot food (S4 Fig). For IL12 expression in tissue samples, male cases showed significant association with age group, habit of smoking, consumption of smoked food, fast food, tobacco, spices, hot food and types of tea consumed (S5 Fig) while female cases showed association with age group, consumption of betel nut, hot food, smoked food, and spices (S6 Fig). While considering blood samples, IL12 expression showed association with age group, habit of smoking, consumption of smoked food, fast food, hot food and spices in male cases (S7 Fig) and female cases were associated with age group, tumor stage, metastasis,

consumption of smoked food and spices and amount of tea consumed (S8 Fig). For IL13 expression, significant associations were observed in histopathology grade, dysphagia grade, location of tumor, consumption of tobacco, hot food and smoked food in tissue samples of male patients (S9 Fig). Among females, significant difference was found in histopathology grade, tumor stage, habit of smoking, consumption of tobacco, fish, meat and smoked food in their tissue samples (S10 Fig). While checking blood samples, male cases showed significant association with histopathology grade, consumption of tobacco, amount of tea consumed and types of tea consumed (S11 Fig) while female cases were found to associate with histopathology grade, consumption of tobacco and khar and differences in the amount of khar consumption (S12 Fig). Association study of IL8, IL12 and IL13 expression with 24 different parameters are listed in Table 2 (for males) and Table 3 (for females).

Table 2. Tabulation of association of IL8, IL12 and IL13 expression with different clinicopathological parameters and lifestyle factors in male ESCC patients.

Clinicopathological parameters and lifestyle factors	No of cases (%)	IL8 tissue		IL8 blood		IL13 tissue		IL13 blood		IL12tissue		IL12 blood	
		mRNA expression	P value	mRNA expression	P value	mRNA expression	P value	mRNA expression	P value	mRNA expression	P value	mRNA expression	P value
Age group:			0.004*		0.001*		0.175		0.297		0.003*		0.012*
<50years	29(41%)	1.70±1.00		1.06±1.27		1.74±0.90		1.69±1.26		1.75±1.09		1.69±1.01	
>50years	41(59%)	2.58±1.35		2.02±1.36		2.10±1.20		2.01±1.31		1.03±0.73		1.12±1.08	
Gender:			0.648		0.539		0.843		0.106		0.571		0.707
Male	70(58%)	2.22±1.29		1.62±1.40		1.95±1.09		1.88±1.29		1.33±0.96		1.33±0.96	
Female	50(42%)	2.33±1.28		1.50±1.37		1.99±1.06		1.48±1.14		1.19±0.78		1.39±0.96	

Location of tumor:			0.655		0.070		0.036*		0.273		0.811		0.918
Upper esophagus	19(97%)	2.14±1.53		1.46±1.32		2.48±0.97		1.69±1.27		1.20±0.83		1.31±1.18	
Middle esophagus	24(34%)	2.08±1.05		1.25±1.25		1.85±1.21		1.72±1.42		1.37±1.00		1.37±1.11	
Lower esophagus	27(39%)	2.40±1.33		2.06±1.51		1.66±0.95		2.15±1.18		1.38±1.02		1.37±1.03	
Histopathology grade:			0.463		0.757		0.003*		0.007*		0.835		0.771
Grade 1:well differentiated ESCC	24(34%)	1.96±0.96		1.68±1.49		1.38±0.80		1.41±1.15		1.26±0.89		1.43±1.20	
Grade 2: moderately differentiated ESCC	35(50%)	2.33±1.40		1.47±1.27		2.14±1.10		1.88±1.27		1.35±1.05		1.27±1.03	
Grade 3:poorly differentiated ESCC	11(16%)	2.44±1.55		1.96±1.65		2.59±1.13		2.89±1.13		1.40±0.87		1.47±1.05	
Dysphagia grade:			0.008*		0.012*		0.026*		0.633		0.214		0.543
Grade 0: Asymptomatic	02(03%)	1.71±2.07		0.64±0.57		2.56±0.99		1.85±1.21		1.17±0.32		0.68±0.93	
Grade 1: Solids with some dysphagia	23(33%)	1.60±1.09		0.81±0.57		2.09±0.85		1.76±1.50		1.52±1.06		1.63±1.16	
Grade 2: Soft, pureed food only	07(10%)	2.01±1.21		1.22±1.10		2.72±1.33		2.06±1.42		1.76±0.99		1.35±0.96	
Grade 3:Liquids only	31(44%)	2.48±1.26		2.09±1.43		1.52±0.99		1.77±1.08		1.13±0.94		1.19±1.07	
Grade 4: No swallowing at all	07(10%)	3.41±1.04		2.86±2.00		2.45±1.44		2.60±1.42		1.16±0.58		1.37±1.11	
Tumor stage:			0.818		0.955		0.552		0.311		0.126		0.789
Stage1	03(04%)	2.45±2.25		1.38±0.21		1.61±0.33		0.69±0.55		2.36±0.51		1.79±1.22	
Stage2	13(19%)	2.17±1.20		1.90±1.80		2.32±0.92		2.00±1.27		1.15±0.74		1.47±1.07	

Stage3	35(50%)	2.10±1.41		1.53±1.18		1.83±1.15		2.04±1.37		1.18±0.83		1.37±1.19	
Stage4	19(27%)	2.44±0.99		1.63±1.62		1.97±1.16		1.69±1.19		1.54±1.24		1.18±0.91	
Node stage:			0.908		0.652		0.650		0.542		0.958		0.314
Stage 0	21(30%)	2.13±1.40		1.75±1.62		1.77±0.85		1.65±1.01		1.29±0.81		1.55±1.33	
Stage1	25(36%)	2.24±1.18		1.44±1.19		2.01±1.12		1.81±1.53		1.30±0.91		1.39±0.96	
Stage2	19(27%)	2.36±1.36		1.77±1.31		1.94±1.35		2.17±1.16		1.31±1.03		1.30±1.01	
Stage3	05(07%)	1.94±1.39		1.35±1.97		2.45±0.78		2.09±1.66		1.67±1.64		0.53±0.48	
Metastasis:			0.036*		0.511		0.702		0.590		0.476		0.095
Absent	54(77%)	2.04±1.27		1.52±1.33		1.92±1.00		1.84±1.29		1.31±0.84		1.49±1.11	
Present	16(33%)	2.81±1.22		1.95±1.61		2.04±1.38		2.01±1.32		1.37±1.32		0.91±0.86	
Smoking:			0.891		0.562		0.573		0.988		0.033*		0.016*
Smokers	39(56%)	2.24±1.34		1.73±1.52		2.01±1.04		1.91±1.42		1.12±0.81		1.12±1.11	
Nonsmokers	31(44%)	2.19±1.25		1.48±1.25		1.86±1.17		1.83±1.12		1.59±1.08		1.65±0.98	
Alcohol:			0.035*		0.573		0.327		0.318		0.764		0.213
Alcoholic	36(51%)	2.53±1.35		1.73±1.51		2.07±1.12		2.03±1.31		1.30±0.98		1.51±1.10	
Nonalcoholic	34(49%)	1.88±1.14		1.50±1.29		1.82±1.06		1.72±1.27		1.36±0.95		1.19±1.06	
Betel nut:			0.002*		0.009*		0.464		0.548		0.821		0.178
Chewers	45(64%)	2.56±1.25		1.98±1.54		2.02±1.08		1.81±1.29		1.38±1.07		1.47±1.10	
Nonchewers	25(36%)	1.60±1.14		0.97±0.79		1.82±1.13		1.32±0.82		1.23±0.73		1.14±1.03	
Tobacco:			0.674		0.013*		0.006*		0.022*		0.032*		0.710
Chewers	46(66%)	2.26±1.37		1.92±1.48		2.20±1.10		2.17±1.40		1.21±0.99		1.37±1.06	
Nonchewers	24(34%)	2.13±1.14		1.05±1.03		1.46±0.91		1.32±0.82		1.56±0.87		1.33±1.15	
Fish:			0.740		0.456		0.522		0.069		0.288		0.648
Consumers	61(87%)	2.20±1.22		1.66±1.41		1.98±1.10		1.98±1.29		1.27±0.93		1.39±1.11	

Nonconsumers	09(13%)	2.35±1.79		1.34±1.37		1.73±1.10		1.19±1.10		1.68±1.11		1.12±0.88	
Meat:			0.008*		0.417		0.635		0.056		0.108		0.283
Consumers	67(96%)	2.30±1.25		1.66±1.42		1.96±1.09		1.81±1.28		1.27±0.92		1.33±1.10	
Nonconsumers	03(04%)	0.32±0.14		0.78±0.63		1.65±1.33		3.31±0.57		2.48±1.24		1.80±0.44	
Egg:			0.392		0.223		0.307		0.337		0.128		0.328
Consumers	62(89%)	2.27±1.30		1.69±1.41		1.90±1.06		1.92±1.26		1.25±0.88		1.32±1.09	
Nonconsumers	08(11%)	1.85±1.18		1.10±1.27		2.32±1.33		1.55±1.55		1.93±1.35		1.59±1.04	
Pickle:			0.304		0.637		0.394		0.256		0.951		0.203
Consumers	53(76%)	2.13±1.19		1.52±1.27		1.88±1.11		1.96±1.27		1.33±0.99		1.26±1.06	
Nonconsumers	17(24%)	2.50±1.56		1.94±1.76		2.15±1.04		1.62±1.37		1.31±0.86		1.64±1.12	
Spices:			0.011*		0.007*		0.840		0.210		0.012*		0.020*
Consumers	53(76%)	2.44±1.33		1.86±1.43		1.96±1.15		1.97±1.23		1.10±0.67		1.14±0.90	
Nonconsumers	17(24%)	1.53±0.86		0.87±1.02		1.90±0.92		1.60±1.48		2.02±1.35		2.02±1.35	
Fast food:			0.500		0.381		0.518		0.788		0.015*		0.007*
Consumers	47(67%)	2.29±1.30		1.67±1.35		1.89±1.13		1.91±1.33		1.12±0.83		1.07±0.86	
Nonconsumers	23(33%)	2.07±1.27		1.52±1.53		2.07±1.03		1.81±1.24		1.74±1.09		1.95±1.26	
Hot food:			0.010*		0.040*		0.043*		0.114		0.014*		0.013*
Consumers	43(61%)	2.53±1.35		1.91±1.52		2.16±1.18		2.07±1.33		1.13±0.88		1.12±1.06	
Nonconsumers	27(39%)	1.72±1.02		1.15±1.05		1.62±0.86		1.57±1.18		1.64±1.01		1.73±1.03	
Type of tea:			0.269		0.036		0.790		0.032*		0.030*		0.199
Red tea	18(26%)	2.15±1.30		1.03±1.14		1.85±0.97		1.30±0.94		1.56±0.97		1.72±1.06	
Milk tea	21(30%)	1.89±1.24		1.98±1.46		2.08±0.95		2.42±1.38		1.03±1.08		1.23±0.98	
Both	31(44%)	2.48±1.30		1.72±1.43		1.92±1.26		1.85±1.29		1.39±0.83		1.23±1.15	
Amount of tea:			0.545		0.269		0.832		0.005*		0.114		0.192

Low	21(30%)	2.29±1.44		1.22±1.21		1.88±1.02		1.14±0.95		1.56±0.86		1.78±1.25	
Medium	24(34%)	2.39±1.03		1.77±1.50		1.90±1.08		2.02±1.21		1.12±0.86		1.15±0.91	
High	25(36%)	1.99±1.39		1.81±1.44		2.06±1.19		2.36±1.38		1.32±1.11		1.19±1.03	
Smoked food:			0.494		0.697		0.013*		0.146		0.017*		0.011*
Consumers	46(66%)	2.29±1.27		1.69±1.46		2.18±1.12		2.04±1.34		1.16±0.96		1.14±1.05	
Nonconsumers	24(34%)	2.07±1.34		1.48±1.30		1.50±0.89		1.57±1.17		1.64±0.89		1.77±1.05	
Khar:			0.883		0.965		0.427		0.413		0.109		0.071
Consumers	47(67%)	2.23±1.19		1.56±1.32		1.87±1.11		1.81±1.35		1.22±0.97		1.20±1.07	
Nonconsumers	23(33%)	2.18±1.50		1.73±1.58		2.10±1.05		2.02±1.18		1.55±0.92		1.68±1.05	
Amount of khar:			0.687		0.850		0.696		0.380		0.121		0.072
No	23(33%)	2.18±1.50		1.73±1.58		2.10±1.05		2.02±1.18		1.55±0.92		1.68±1.05	
Low	23(33%)	2.47±1.13		1.61±1.28		1.74±1.21		1.63±1.22		1.31±0.75		1.06±082	
Medium	09(13%)	1.99±1.39		1.84±1.66		1.90±1.15		2.33±1.15		0.95±0.50		0.70±0.57	
High	15(21%)	2.02±1.15		1.33±1.21		2.07±0.97		1.78±1.62		1.25±1.41		1.71±1.45	

Note: Data are represented as mean ± standard deviation. * Statistically significant p value.

Table 3. Tabulation of association of IL8, IL12 and IL13 expression with different clinicopathological parameters and lifestyle factors in female ESCC patients.

Clinicopathological parameters and lifestyle factors	No of cases (%)	IL8 tissue		IL8 blood		IL13 tissue		IL13 blood		IL12 tissue		IL12 blood	
		mRNA expression	P value	mRNA expression	P value	mRNA expression	P value	mRNA expression	P value	mRNA expression	P value	mRNA expression	P value
Age group:			0.002*		0.035*		0.394		0.777		0.009*		0.037*
<50years	18(36%)	1.61±1.21		1.03±1.26		1.81±1.22		1.34±1.03		1.56±0.74		1.76±1.03	
>50years	32(64%)	2.73±1.15		1.77±1.38		2.09±0.97		1.55±1.21		0.97±0.73		1.19±0.86	
Location of tumor:			0.655		0.612		0.261		0.133		0.948		0.217
Upper esophagus	19(38%)	2.43±1.30		1.31±1.23		1.72±0.98		1.17±0.98		1.16±0.81		1.44±1.06	
Middle esophagus	21(42%)	2.13±1.25		1.39±1.17		2.04±1.03		1.55±1.28		1.23±0.74		1.53±0.76	
Lower esophagus	10(20%)	2.54±1.38		2.11±1.92		2.40±1.24		1.89±1.06		1.14±0.87		1.01±1.11	
Histopathology grade:			0.810		0.591		0.019*		0.005*		0.559		0.858
Grade 1:well differentiated ESCC	12(24%)	2.12±1.13		1.37±1.24		1.65±0.79		0.84±0.52		1.35±0.86		1.52±1.12	
Grade 2: moderately differentiated ESCC	30(60%)	2.37±1.40		1.70±1.50		1.87±1.06		1.35±0.88		1.18±0.83		1.37±0.93	
Grade 3:poorly differentiated ESCC	08(16%)	2.48±1.14		0.96±0.97		2.92±1.03		2.89±1.57		0.95±0.35		1.29±0.90	
Dysphagia grade:			0.020*		0.045*		0.074		0.438		0.981		0.052
Grade 0: Asymptomatic	02(04%)	0.82±0.26		0.56±0.69		1.11±0.56		3.32±2.20		1.00±0.04		2.45±0.01	
Grade 1: Solids with some dysphagia	15(30%)	1.87±1.19		0.83±0.79		1.93±0.80		1.48±1.23		1.27±0.92		1.75±0.92	
Grade 2: Soft, pureed food only	11(22%)	2.24±1.24		1.18±0.78		1.82±1.18		1.25±1.04		1.12±0.72		1.50±1.16	

Grade 3:Liquids only Grade 4: No swallowing at all	15(30%) 07(14%)	2.49±128 3.52±0.78		2.04±1.60 2.55±1.81		1.82±1.21 3.00±0.62		1.37±0.77 1.54±1.36		1.20±0.77 1.11±0.82		1.13±0.75 0.72±0.72	
Tumor stage: Stage 1 Stage 2 Stage 3 Stage 4	 02(04%) 13(26%) 27(54%) 08(16%)	 0.99±1.05 1.73±1.17 2.29±1.19 3.76±0.48	0.001*	 0.13±0.06 0.73±0.66 1.57±1.23 2.87±1.75	0.005*	 0.68±0.19 1.60±0.86 1.96±1.05 3.05±0.71	0.003*	 1.41±0.08 1.79±1.54 1.29±0.91 1.62±1.28	0.788	 1.90±0.03 1.25±0.71 1.26±0.85 0.66±0.43	0.128	 2.83±0.64 1.72±0.91 1.30±0.93 0.80±0.70	0.035*
Node stage: Stage 0 Stage 1 Stage 2 Stage 3	 11(22%) 22(44%) 12(22%) 06(12%)	 2.03±1.36 2.20±1.33 2.67±1.00 2.70±1.51	0.574	 1.67±1.24 1.06±1.10 1.84±1.71 2.20±1.65	0.312	 1.65±0.91 1.89±1.05 2.13±1.17 2.69±1.06	0.258	 1.02±0.52 0.57±1.02 1.85±1/73 1.29±1.02	0.646	 1.61±0.82 0.94±0.78 1.28±0.59 1.13±0.81	0.136	 1.60±1.12 1.41±0.92 1.32±0.98 1.08±0.84	0.814
Metastasis: Absent Present	 36(72%) 14(28%)	 2.21±1.25 2.63±1.35	0.298	 1.24±1.23 2.16±1.53	0.066	 1.87±1.04 2.29±1.09	0.213	 1.41±1.21 1.64±0.95	0.191	 1.19±0.77 1.18±0.83	0.987	 1.58±0.96 0.91±0.78	0.035*
Smoking: Smokers Nonsmokers	 09(18%) 41(82%)	 2.60±1.40 2.27±1.26	0.495	 1.58±1.44 1.48±1.38	0.733	 2.76±0.70 1.82±1.06	0.015*	 1.57±1.14 1.46±1.15	0.570	 0.88±0.98 1.25±0.73	0.203	 1.03±0.93 1.47±0.95	0.283
Alcohol: Alcoholic Nonalcoholic	 05(10%) 45(90%)	 3.09±1.36 2.24±1.26	0.161	 1.79±1.81 1.47±1.34	0.686	 2.83±0.84 1.89±1.05	0.063	 1.31±0.59 1.50±1.19	0.771	 0.71±0.53 1.24±0.79	0.159	 1.01±0.96 1.44±0.96	0.438
Betel nut: Chewers	 36(72%)	 2.56±1.30	0.041*	 1.77±1.45	0.023*	 1.97±1.14	0.879	 1.60±1.21	0.199	 1.02±0.73	0.014*	 1.34±0.94	0.604

Nonchewers	14(28%)	1.73±1.06		0.80±0.83		2.03±0.86		1.15±0.88		1.62±0.77		1.52±1.03	
Tobacco:			0.690		0.821		0.024*		0.017*		0.082		0.284
Chewers	29(58%)	2.39±1.24		1.44±1.29		2.27±1.14		1.83±1.28		1.02±0.82		1.26±0.93	
Nonchewers	21(42%)	2.24±1.37		1.58±1.50		1.59±0.82		0.99±0.68		1.41±0.67		1.58±0.96	
Fish:			0.754		0.482		0.022*		0.065		0.266		0.438
Consumers	40(80%)	2.36±1.28		1.56±1.36		2.16±1.09		1.62±1..21		1.12±0.79		1.34±0.92	
Nonconsumers	10(20%)	2.21±1.37		1.26±1.46		1.30±0.60		0.89±0.53		1.43±0.74		1.62±1.09	
Meat:			0.824		0.615		0.035*		0.078		0.247		0.128
Consumers	43(86%)	2.34±1.28		1.46±1.36		2.12±1.09		1.59±1.17		1.37±0.77		1.31±0.95	
Nonconsumers	07(14%)	2.22±1.40		1.73±1.56		1..20±0.37		0.79±0.60		1.51±0.82		1.88±0.94	
Egg:			0.321		0.884		0.439		0.961		0.800		0.910
Consumers	45(90%)	2.27±1.29		1.48±1.32		2.03±1.06		1.48±1.14		1.19±0.80		1.39±0.98	
Nonconsumers	05(10%)	2.87±1.17		1.70±2.00		1.63±1.19		1.48±1.22		1.10±0.59		1.44±0.76	
Pickle:			0.804		0.382		0.856		0.578		0.628		0.508
Consumers	42(84%)	2.35±1.38		1.59±1.43		1.98±1.10		1.45±1.16		1.21±0.79		1.43±0.98	
Nonconsumers	08(16%)	2.22±0.55		1.03±0.94		2.05±0.88		1.62±1.11		1.06±0.73		1.18±0.84	
Spices:			0.031*		0.041*		0.962		0.261		0.004*		0.034*
Consumers	36(72%)	2.57±1.27		1.76±1.48		1.98±1.10		1.62±1.24		0.99±0.65		1.20±0.93	
Nonconsumers	14(28%)	1.70±1.13		0.83±0.75		2.00±1.00		1.12±0.73		1.68±0.89		1.88±0.87	
Fast food:			0.976		0.674		0.374		0.324		0.427		0.860
Consumers	22(44%)	2.32±1.33		1.63±1.49		2.14±0.98		1.67±1.23		1.08±0.70		1.41±095	
Nonconsumers	28(56%)	2.33±1.27		1.40±1.29		1.79±1.14		1.33±1.06		1.26±0.84		1.38±0.98	
Hot food:			0.022*		0.033*		0.203		0.547		0.034*		0.062
Consumers	25(50%)	2.74±1.26		1.97±1.49		2.18±0.97		1.36±1.01		0.95±0.73		1.14±0.88	

Nonconsumers	25(50%)	1.92±1.19		1.04±1.08		1.79±1.14		1.60±1.27		1.42±0.77		1.65±0.98	
Type of tea:			0.189		0.595		0.149		0.512		0.133		0.175
Red tea	08(16%)	2.43±1.02		1.08±1.50		1.65±0.97		1.21±1.03		1.61±0.69		1.37±1.05	
Milk tea	21(42%)	1.95±1.51		1.56±1.29		1.77±1.04		1.80±1.43		1.24±0.88		1.70±0.99	
Both	21(42%)	2.67±1.04		1.60±1.44		2.33±1.07		1.26±0.75		0.97±0.65		1.10±0.83	
Amount of tea:			0.011*		0.594		0.248		0.689		0.647		0.047*
Low	20(40%)	2.63±1.21		1.62±1.52		1.95±1.05		1.46±1.08		1.26±0.92		1.51±0.92	
Medium	15(30%)	1.52±1.14		1.23±1.10		1.68±0.78		1.35±1.20		1.24±0.67		1.75±1.04	
High	15(30%)	2.73±1.21		1.61±1.46		2.34±1.27		1.63±1.22		1.03±0.70		0.89±0.73	
Smoked food:			0.042*		0.938		0.037*		0.837		0.022*		0.029*
Consumers	28(56%)	2.65±1.35		1.57±1.46		2.27±1.07		1.53±1.28		0.96±0.72		1.12±0.86	
Nonconsumers	22(44%)	1.91±1.08		1.41±1.29		1.63±0.97		1.41±0.96		1.47±0.77		1.75±0.97	
Khar:			0.282		0.409		0.593		0.019*		0.360		0.081
Consumers	35(70%)	2.46±1.33		1.60±1.41		1.93±1.11		1.73±1.25		1.12±0.80		1.26±0.97	
Nonconsumers	15(30%)	2.02±1.13		1.28±1.32		2.11±0.97		0.89±0.45		1.34±0.73		1.72±0.86	
Amount of khar:			0.655		0.721		0.219		0.007*		0.641		0.148
No	15(30%)	2.02±1.13		1.28±1.32		2.11±0.97		0.89±0.45		1.34±0.73		1.72±0.86	
Low	17(34%)	2.46±1.41		1.59±1.45		1.74±0.98		1.22±0.72		1.13±0.84		1.50±1.10	
Medium	09(18%)	2.66±1.32		2.01±1.59		2.21±0.96		1.47±1.04		0.93±0.79		0.99±0.87	
High	09(18%)	2.24±1.32		1.19±1.14		2.03±1.49		2.94±1.53		1.28±0.78		1.05±0.78	

Note: Data are represented as mean ± standard deviation. * Statistically significant p value.

Relationship between IL8, IL12 and IL13 expression in tissue and blood samples of ESCC patients

Significant positive correlation ($p<0.05$) was observed between tissue and blood level expression of IL8, IL12 and IL13**.** Again, a significant positive correlation of IL8 and IL13 expression and a significant negative correlation of IL8 and IL12 expression were revealed when correlation was performed between the two cytokines in both tissue and blood level. Correlation study of IL8, IL12 and IL13 gene expression in tissue and blood level are represented in Table 4 and S13 Fig.

Table 4. Correlation study of IL8, IL12 and IL13 gene expression in tissue and blood level.

Parameters	P value	Correlation
IL8 tissue and IL8 blood	0.000*	0.492
IL13 tissue and IL13 blood	0.017*	0.217
IL12 tissue and IL12 blood	0.004*	0.262
IL8 tissue and IL13 tissue	0.047*	0.182
IL8 tissue and IL12 tissue	0.000*	-0.372
IL13 tissue and IL12 tissue	0.082	-0.159
IL8 blood and IL13 blood	0.001*	0.301
IL8 blood and IL12 blood	0.023*	-0.208
IL13 blood and IL12 blood	0.651	-0.042

Note: * Statistically significant p value.

Survival analysis

Among males, tumor stage 4 was observed with lower mean survival time than other tumor stages (p=0.012). Similarly, males possessing node stage 3 were noted with lower survival time than other node stages (p=0.009). Metastatic male patients were observed to have a lower survival than nonmetastatic males (p=0.024). Males consuming alcohol, hot food and smoked food in their diet were noticed with lower survival time than the others who don't consume the same (p=0.021, p=0.013 and p=0.021 respectively). Again, males having lower levels of IL12 in their tissue were observed to have lower survival compared to males with higher IL12 expression (p=0.005).

Females possessing histology grade 3 were noticed to have lower survival compared to histopathology grade 1 and 2 (p=0.024). Similarly, females possessing tumor stage 1 were noted with higher mean survival time than other tumor stages (p=0.015). Metastatic females were observed with a lower survival time than non-metastatic one (p=0.022). Females consuming betel nut, tobacco, spices, hot food and smoked food were noticed with lower survival time than the others who don't consume the same (p=0.030, p=0.015, p=0.015, p=0.004 and p=0.015 respectively). Females having lower levels of IL12 in their tissue and blood were observed to have lower survival compared to females with higher IL12 expression in their tissue and blood (p=0.003 and p=0.004 respectively). Similarly, females having higher levels of IL8 expression in their blood and higher levels of IL13 expression in their tissue were noted to have lower survival (p=0.016 and p=0.031 respectively). Survival analysis data for males and females are listed in Table 5.

Table 5. Tabulation of survival analysis data of both male and female ESCC patients.

Parameters	Groups	Male					Female				
		Mean estimate (in months)	Standard error	95% confidence interval		p value	Mean estimate (in months)	Standard error	95% confidence interval		p value
				Lower bound	Upper bound				Lower bound	Upper bound	
Age group	<50yrs	17.677	1.676	14.392	20.961	0.568	16.806	2.602	11.705	21.906	0.737
	>50yrs	16.771	1.252	14.317	19.225		15.972	1.518	12.996	18.948	
Gender	Male	17.391	1.081	15.273	19.509	0.616					
	Female	16.396	1.387	13.677	19.116						
Location of tumor	Upper	18.003	1.853	14.370	21.635	0.606	18.425	2.487	13.550	23.300	0.440
	Middle	17.500	1.741	14.088	20.912		14.683	1.546	11.653	17.712	
	Lower	16.111	1.625	12.926	19.296		15.200	3.139	9.047	21.353	
Histopathology grade	Grade 1	19.631	2.005	15.702	23.560	0.341	21.292	2.814	15.777	26.806	0.024*
	Grade 2	15.743	1.255	13.283	18.203		15.840	1.706	12.496	19.183	
	Grade 3	16.182	2.221	11.830	20.534		9.500	1.132	7.281	11.719	
Dysphagia grade	Grade 0	16.000	0.000	16.000	16.000	0.811	16.500	3.500	9.640	23.360	0.747
	Grade 1	19.000	1.735	15.599	22.401		19.267	2.499	14.369	24.164	
	Grade 2	17.857	3.120	11.743	23.972		15.273	3.372	8.663	21.883	
	Grade 3	15.710	1.671	12.434	18.985		14.067	1.944	10.256	17.877	
	Grade 4	15.714	1.156	13.449	17.979		13.857	2.773	8.423	19.291	
Tumor stage	Stage 1	27.667	7.313	13.333	42.000	0.012*	34.500	2.475	29.649	39.351	0.015*
	Stage 2	21.811	2.435	17.039	26.584		18.356	2.095	14.249	22.462	
	Stage 3	16.800	1.283	14.285	19.315		15.543	1.893	11.832	19.254	
	Stage 4	13.263	1.713	9.905	16.621		10.375	1.117	8.186	12.564	
Node stage	Stage 0	18.943	1.981	15.059	22.827	0.009*	21.000	3.168	14.791	27.209	0.125
	Stage 1	18.000	1.558	14.946	21.054		15.234	1.694	11.914	18.553	
	Stage 2	15.842	1.858	12.200	19.484		16.727	3.296	10.268	23.187	
	Stage 3	10.000	1.483	7.093	12.907		10.667	2.940	4.904	16.429	
Metastasis	Absent	18.518	1.320	15.930	21.106	0.024*	18.283	1.627	15.093	21.473	0.022*
	Present	13.688	1.287	11.165	16.210		11.714	2.309	7.188	16.241	
Betel nut	Non chewers	18.772	1.791	15.262	22.283	0.125	22.214	2.700	16.921	27.507	0.030*
	chewers	16.277	1.204	13.916	18.637		14.071	1.394	11.339	16.804	
Tobacco	Non chewers	18.242	1.615	15.075	21.408	0.562	20.419	2.088	16.327	24.511	0.015*
	chewers	16.758	1.334	14.142	19.373		13.353	1.572	10.272	16.435	
Alcohol	Non alcoholic	19.991	1.787	16.489	23.494	0.021*	16.665	1.531	13.664	19.666	0.517
	Alcoholic	14.938	1.110	12.762	17.113		14.200	1.655	10.956	17.444	

Smoking	Non smokers	17.728	1.675	14.445	21.012	0.332	16.893	1.666	13.627	20.158	0.373
	Smokers	16.744	1.237	14.320	19.167		14.444	1.547	11.413	17.476	
Meat	Non consumers	17.667	6.672	4.589	30.744	0.557	24.429	3.288	17.984	30.873	0.052
	Consumers	17.254	1.055	15.186	19.321		15.035	1.392	12.306	17.764	
Fish	Non consumers	19.333	3.103	13.251	25.416	0.386	22.640	3.321	16.131	29.149	0.068
	Consumers	17.018	1.103	14.855	19.180		14.847	1.409	12.085	17.608	
Egg	Non consumers	15.500	2.383	10.829	20.171	0.447	19.400	3.250	13.031	25.769	0.677
	Consumers	17.659	1.187	15.332	19.986		16.105	1.515	13.135	19.075	
Hot food	Non consumers	20.889	1.818	17.327	24.451	0.013*	20.151	2.089	16.058	24.245	0.004*
	Consumers	14.819	1.066	12.729	16.908		12.300	1.242	9.866	14.734	
Smoked food	Non consumers	20.852	2.062	16.810	24.894	0.021*	20.119	2.280	15.649	24.588	0.015*
	Consumers	15.242	1.012	13.259	17.226		13.170	1.366	10.492	15.848	
Fast food	Non consumers	18.783	1.607	15.633	21.932	0.279	17.881	2.001	13.960	21.802	0.209
	Consumers	16.642	1.385	13.928	19.356		14.364	1.695	11.041	17.687	
Spices	Non consumers	19.941	2.104	15.818	24.064	0.191	22.504	3.031	16.564	28.443	0.015*
	Consumers	16.523	1.228	14.115	18.931		14.052	1.338	11.429	16.676	
Pickle	Non consumers	18.250	1.469	15.370	21.130	0.851	18.458	2.753	13.062	23.855	0.419
	Consumers	17.083	1.339	14.458	19.707		15.788	1.495	12.859	18.718	
Type of tea	Red tea	18.050	1.909	14.309	21.791	0.821	18.375	3.732	11.061	25.689	0.815
	Milk tea	17.524	2.117	13.374	21.674		15.603	1.861	11.956	19.251	
	Both	16.419	1.383	13.708	19.131		16.488	2.413	11.758	21.218	
Amount of tea	Low	19.810	2.005	15.880	23.739	0.069	18.491	2.350	13.885	23.097	0.122
	Medium	17.865	1.873	14.193	21.536		17.067	2.549	12.071	22.062	
	High	14.472	1.367	11.793	17.151		12.400	1.473	9.512	15.288	
Khar	Non consumers	17.576	1.481	14.674	20.479	0.715	16.422	2.479	11.563	21.282	0.962
	Consumers	17.113	1.375	14.417	19.808		16.419	1.688	13.111	19.726	
Amount of khar	No	17.576	1.481	14.674	20.479	0.733	16.422	2.479	11.563	21.282	0.309
	Low	17.417	2.236	13.034	21.800		19.566	2.969	13.748	25.385	
	Medium	14.556	0.973	12.648	16.463		13.667	2.273	9.212	18.122	
	High	17.800	2.273	13.345	22.255		14.000	2.804	8.505	19.495	
IL8 expression in tissue	Low	21.528	3.081	15.490	27.566	0.117	17.875	3.646	10.728	25.022	0.645
	High	16.480	1.056	14.409	18.550		15.938	1.431	13.133	18.743	
IL8 expression in blood	Low	19.170	1.506	16.218	22.123	0.243	20.883	2.412	16.156	25.610	0.016*
	High	16.166	1.420	13.382	18.949		13.527	1.414	10.756	16.298	
IL13 expression in tissue	Low	16.909	2.870	11.283	22.535	0.647	22.611	4.345	14.096	31.127	0.031*
	High	17.291	1.105	15.126	19.456		14.848	1.226	12.444	17.251	

IL13 expression in blood	Low	20.364	1.929	16.584	24.144	0.070	17.647	2.229	13.278	22.016	0.565
	High	15.834	1.199	13.484	18.184		15.634	1.690	12.322	18.947	
IL12 expression in tissue	Low	14.154	1.108	11.983	16.325	0.005*	11.550	1.083	9.428	13.672	0.003*
	High	19.398	1.539	16.381	22.414		19.353	1.921	15.587	23.119	
IL12 expression in blood	Low	15.750	1.337	13.130	18.370	0.161	12.591	1.262	10.118	15.064	0.004*
	High	18.505	1.544	15.479	21.530		19.601	2.165	15.357	23.845	

Note: * Statistically significant p value.

Hazard analysis

The hazard (mortality) ratio is 4.649 times higher for males having node stage 3 compared to node stage 0 (p=0.004). The hazard ratio is higher for males having metastasis compared to nonmetastatic one (p=0.036). Similarly, the hazard ratio is higher for males consuming alcohol, hot food and smoked food in their diet compared to one who don't consume the same (p=0.032, p=0.021 and p=0.032 respectively). The hazard ratio is higher for males consuming higher amount of tea compared to one consuming lower amount of tea (p=0.037). Moreover, the hazard ratio is 0.499 times for male patients with higher levels of IL12 in their tissue compared to one with a lower level of IL12 expression (p=0.009).

Among females, significant difference in hazard ratio was observed between histopathology grade 1 and histopathology grade 3 (p=0.012). The hazard ratio is higher for females having tumor stage 4 compared to tumor stage 1 (p=0.014). Similarly, the hazard ratio is higher for females having node stage 3 compared to node stage 0 (p=0.029). The hazard ratio for a metastatic female patient is 2.064 times that of a nonmetaststic one (P=0.032). The hazard ratio is significantly higher in females consuming betel nut, tobacco, hot food, smoked food and spices compared to others who don't consume the same (p=0.042, p=0.023, p=0.007, p=0.023 and p=0.023 respectively). Moreover, the hazard ratio is higher for female patients

with higher levels of IL8 expression in their blood samples and higher levels of IL13 expression in their tissue samples (p=0.023 and p=0.045 respectively). Again, the hazard ratio is 0.404 times and 0.425 times for female patients with higher levels of IL12 in their tissue and blood compared to one with a lower level of IL12 expression (p=0.007 and p=0.008 respectively). Hazard analysis data for males and females are listed in Table 6. Survival and hazard analysis graphs representing different parameters are presented in S14-S20 Figs for male patients and S21-S28 Figs for female patients.

Table 6. Tabulation of hazard analysis data of both male and female ESCC patients.

Parameters	Group1/group2	Male				Female			
		p value	Hazard Ratio	95% Confidence interval		p value	Hazard Ratio	95% Confidence interval	
				Lower	Upper			Lower	Upper
Age group	>50yrs/<50yrs	0.591	1.149	0.692	1.906	0.748	1.111	0.585	2.110
Gender	Female/Male	0.633	1.099	0.745	1.621				
Location of tumor	Middle/Upper	0.843	1.069	0.553	2.067	0.228	1.540	0.763	3.110
	Lower/Upper	0.397	1.318	0.696	2.494	0.492	1.340	0.582	3.084
Histopathology grade	Grade 2/ Grade 1	0.172	1.470	0.845	2.558	0.201	1.629	0.772	3.437
	Grade 3/ Grade 1	0.480	1.328	0.604	2.920	0.012*	3.773	1.340	10.617
Dysphagia grade	Grade 1/ Grade 0	0.932	1.091	0.145	8.204	0.587	0.658	0.145	2.977
	Grade 2/ Grade 0	0.966	1.047	0.125	8.809	0.809	0.825	0.174	3.923
	Grade 3/ Grade 0	0.703	1.475	0.199	10.931	0.999	1.001	0.223	4.488
	Grade 4/ Grade 0	0.848	1.230	0.147	10.278	0.864	1.149	0.235	5.617
Tumor stage	Stage 2/ Stage 1	0.655	1.416	0.309	6.498	0.110	5.694	0.676	47.992
	Stage 3/ Stage 1	0.236	2.380	0.567	9.986	0.072	6.642	0.845	52.193
	Stage 4/ Stage 1	0.062	4.056	0.931	17.668	0.014*	15.664	1.745	140.609
Node stage	Stage 1 / Stage 0	0.771	1.097	0.588	2.048	0.165	1.816	0.783	4.214
	Stage 2 / Stage 0	0.234	1.491	0.772	2.880	0.440	1.458	0.560	3.792
	Stage 3 / Stage 0	0.004*	4.649	1.643	13.152	0.029*	3.358	1.132	9.955
Metastasis	Present / Absent	0.036*	1.870	1.043	3.351	0.032*	2.064	1.064	4.006
Betel nut	Consumers / Non consumers	0.152	1.475	0.867	2.511	0.042*	2.114	1.028	4.348

Tobacco	Consumers / Non consumers	0.586	1.157	0.684	1.959	0.023*	2.082	1.109	3.911
Alcohol	Consumers / Non consumers	0.032*	1.740	1.048	2.891	0.540	1.347	0.520	3.486
Smoking	Consumers / Non consumers	0.363	1.264	0.763	2.095	0.397	1.385	0.651	2.947
Meat	Consumers / Non consumers	0.583	1.490	0.358	6.200	0.071	2.385	0.930	6.121
Fish	Consumers / Non consumers	0.416	1.419	0.611	3.297	0.087	2.037	0.902	4.599
Egg	Consumers / Non consumers	0.475	0.762	0.362	1.606	0.692	1.209	0.472	3.101
Hot food	Consumers / Non consumers	0.021*	1.848	1.095	3.120	0.007*	2.433	1.272	4.654
Smoked food	Consumers / Non consumers	0.032*	1.807	1.052	3.104	0.023*	2.108	1.107	4.017
Fast food	Consumers / Non consumers	0.311	1.311	0.777	2.213	0.232	1.452	0.788	2.676
Spices	Consumers / Non consumers	0.223	1.432	0.804	2.551	0.023*	2.367	1.123	4.985
Pickle	Consumers / Non consumers	0.826	1.067	0.600	1.895	0.442	1.406	0.590	3.349
Type of tea	Milk / Red tea	0.800	1.092	0.553	2.157	0.576	1.270	0.549	2.937
	Both milk and red / Red tea	0.563	1.202	0.644	2.246	0.836	1.095	0.462	2.593
Amount of tea	Medium / Low	0.515	1.231	0.659	2.301	0.577	1.233	0.591	2.571
	High / Low	0.037*	1.933	1.040	3.592	0.058	2.074	0.975	4.412
Khar	Consumers / Non consumers	0.731	1.099	0.643	1.878	0.963	0.985	0.513	1.890
Amount of khar	Low / No	0.941	1.024	0.547	1.917	0.365	0.695	0.316	1.527
	Medium / No	0.318	1.500	0.677	3.321	0.484	1.360	0.574	3.222
	High / No	0.979	1.009	0.504	2.021	0.469	1.372	0.583	3.233
IL8 expression in tissue	High / Low	0.145	1.717	0.830	3.552	0.658	1.202	0.531	2.720
IL8 expression in blood	High / Low	0.274	1.329	0.798	2.214	0.023*	2.166	1.112	4.218
IL13 expression in	High / Low	0.667	1.168	0.575	2.372	0.045*	2.685	1.021	7.061

tissue									
IL13 expression in blood	High / Low	0.091	1.600	0.927	2.759	0.582	1.198	0.630	2.278
IL12 expression in tissue	High / Low	0.009*	0.499	0.297	0.838	0.007*	0.404	0.210	0.776
IL12 expression in blood	High / Low	0.188	0.713	0.431	1.179	0.008*	0.425	0.226	0.797

Note: * Statistically significant p value.

Discussion

This study targeted both tissue and blood samples of the targeted patients for analysis of expression profile of the selected cytokines. This study of gene expression at the blood and tissue levels was done independently for men and women. Till date very less expression studies were performed together in both tissue and blood samples.

High level of IL8 expression was observed in many human cancers, including breast, lung, prostate, pancreatic, colorectal, esophageal cancers as well as melanoma and numerous studies have shown that serum level of IL-8 in cancer patients can act like a prognostic marker [21-25]. Increased IL8 expression was observed in gastric cancer tissue and overexpression of IL-8 was reported to have association with prognosis of this cancer [26]. Higher level of IL8 was also reported in the sera of liver, gastric and non-small-cell lung cancer patients as compared to healthy controls [27]. Moreover, overexpression of IL8 was correlated with tumor progression, recurrence and the TNM stage in multiple cancers [28]. In our study, 83% patients showed overexpression of IL8 in their tissue (p=0.000). This data reveal higher potential of IL8 expression in tissue samples as prospective molecular biomarker for screening ESCC. But only 62% patients were noted to have higher levels of

IL8 expression in their blood (p=0.388). Moreover, a significant positive correlation (p=0.000) between blood and tissue level IL8 expression was also observed in this study.

Higher level of IL12 was reported in the sera of non-small-cell lung cancer, prostate carcinoma and metastatic renal cell carcinoma patients as compared to healthy controls [27, 29]. But lower level of IL12 was reported in the serum of breast cancer patients when compared to healthy controls [30]. Higher level of IL12 was also reported in the serum of esophageal SCC patients as compared to controls [31]. In our study, high level of IL12 expression was observed in 57% blood samples (p=0.222) and 62% tissue samples (p=0.435). A significant positive correlation (p=0.004) between blood and tissue level IL12 expression was also observed in ESCC patients.

Increased expression of IL13 was reported in peripheral blood of breast, prostate and bladder cancer patients [32]. High level of IL13 expression was noticed in pancreatic cancer, lymphoma, oral squamous cell carcinoma and non-small cell lung Carcinoma patients [33-36]. Plasma level of IL-13 was significantly higher in bladder cancer patients than in the healthy controls and higher serum IL-13 level was reported to have association with progression of diffuse large B cell lymphoma [37, 38]. Higher concentration of IL-13 was observed in colorectal and upper gastrointestinal tract tumors than adjacent normal tissue [39]. Higher level of IL13 was also reported in the sera of melanoma and skin cancer patients as compared to healthy controls [27]. In our study, high level of IL13 expression was observed in 68% blood samples (p=0.312) and 83% tissue samples (p=0.001). These data indicate a higher potential of IL13 expression in tissue samples as prospective molecular biomarker for screening ESCC. Additionally, a significant positive correlation (p=0.017) between blood and tissue level IL13 expression was also observed in this study.

In India, the role of diet, nutrition and food habits in causing esophageal cancer has been given attention recently. Consumption of very hot foods, spices, smoked food and some locally made food e.g. khar (kalakhar), a locally made food of Assam, were reported to have significant associations with the risk esophageal cancer development [40-42]. Betel nut chewing, consumption of tobacco (smoking and smokeless tobacco) and alcohol were noted to have significant association with an increased risk of esophageal cancer [40, 42, 43]. In our study, different clinicopathological factors and dietary habits like age group, dysphagia grade, histopathology grade, consumption of betel nut, tobacco, hot food, smoked food, spices, etc. showed significant association ($p<0.05$) with IL8, IL13 and IL12 expression in tissue and blood samples of ESCC patients. These data represent their association and clinical significance with the alteration of the studied cytokines' expression in ESCC.

Although esophageal cancer prevention approaches are essential, measures to lower morbidity or increase survival are equally crucial. Different clinicopathological parameters and risk factors like histopathology grade, age, gender, tumor stage etc. affect the survival of ESCC patients [20, 44, 45]. In our study, tumor stage, node stage, metastasis, consumption of hot food, smoked food and alcohol showed significant association ($p<0.05$) with survival of male patients, whereas survival of female patients showed association ($p<0.05$) with histopathology grade, tumor stage, metastasis, consumption of betel nut, spices, smoked food, hot food and tobacco. Moreover, the univariate model of hazard analysis data also supports these findings and all these data represent their clinical importance for detecting survival of ESCC patients. When the expression of IL8, IL13, and IL12 was analysed with survival and hazard outcomes, statistically significant association was observed in IL8 expression in female patient blood, IL13 expression in female patient tissue, and IL12 expression in male and female patient tissue as well as in female patient blood. This clearly illustrates the

function of these cytokines and their clinical significance in determining ESCC patient survival.

The findings of our study show some similarities and differences with those of other studies, which may be caused by variations in sample size, geographic location, genetic and environmental factors, racial and ethnic diversity, associated clinical conditions, etc. The distinct genetic makeup and indigenous food habit among the Northeast Indian population may be factors for higher incidence of esophageal cancer in this area. Different risk factors or variables like individual diet, nutrition, lifestyle, food habits, genetic, epigenetic and environmental factors, etc. determine which type of cancer predominates in a given patient or in a given geographical location [40, 42, 46]. This study will provide us to acquire more knowledge towards the role of IL8, IL12 and IL13 on ESCC progression along with its interaction with different clinicopathological factors and dietary habits for causing this cancer in the Northeast Indian population.

In conclusion, altered expression of IL8, IL12 and IL13 may be associated with ESCC progression. This expression study also reveals the correlation of studied cytokines in tissue and blood level and the association of different clinicopathological and dietary factors in ESCC. Moreover, both survival and hazard analysis data also reveals the impact of different factors on survival and mortality rate of ESCC patients. Again, overexpression of both IL8 and IL13 in tissue samples may be a potential biomarker for ECSS screening among Northeast Indian Population. This type of gene expression study along with survival and hazard outcomes will enable us to learn more about and develop a deeper understanding of the biology of esophageal cancer.

Supporting information

S1 Fig. Box plot representation of significant association (p<0.05) of IL8 expression in tissue level with different parameters in ESCC male patients.

S2 Fig. Box plot representation of significant association (p<0.05) of IL8 expression in tissue level with different parameters in ESCC female patients.

S3 Fig. Box plot representation of significant association (p<0.05) of IL8 expression in blood level with different parameters in ESCC male patients.

S4 Fig. Box plot representation of significant association (p<0.05) of IL8 expression in blood level with different parameters in ESCC female patients.

S5 Fig. Box plot representation of significant association (p<0.05) of IL12 expression in tissue level with different parameters in ESCC male patients.

S6 Fig. Box plot representation of significant association (p<0.05) of IL12 expression in tissue level with different parameters in ESCC female patients.

S7 Fig. Box plot representation of significant association (p<0.05) of IL12 expression in blood level with different parameters in ESCC male patients.

S8 Fig. Box plot representation of significant association (p<0.05) of IL12 expression in blood level with different parameters in ESCC female patients.

S9 Fig. Box plot representation of significant association (p<0.05) of IL13 expression in tissue level with different parameters in ESCC male patients.

S10 Fig. Box plot representation of significant association (p<0.05) of IL13 expression in tissue level with different parameters in ESCC female patients.

S11 Fig. Box plot representation of significant association (p<0.05) of IL13 expression in blood level with different parameters in ESCC male patients.

S12 Fig. Box plot representation of significant association ($p<0.05$) of IL13 expression in blood level with different parameters in ESCC female patients.

S13 Fig. Scattered plot representation of correlation of IL8, IL12 and IL13 expression in tissue and blood level

S14 Fig. Graphical representation of survival and hazard analysis in males representing different parameters.

S15 Fig. Graphical representation of survival and hazard analysis in males representing IL8 expression in tissue level.

S16 Fig. Graphical representation of survival and hazard analysis in males representing IL8 expression in blood level.

S17 Fig. Graphical representation of survival and hazard analysis in males representing IL12 expression in tissue level.

S18 Fig. Graphical representation of survival and hazard analysis in males representing IL12 expression in blood level.

S19 Fig. Graphical representation of survival and hazard analysis in males representing IL13 expression in tissue level.

S20 Fig. Graphical representation of survival and hazard analysis in males representing IL13 expression in blood level.

S21 Fig. Graphical representation of survival and hazard analysis in females representing different parameters.

S22 Fig. Graphical representation of survival and hazard analysis in ESCC patients representing gender.

S23 Fig. Graphical representation of survival and hazard analysis in females representing IL8 expression in tissue level.

S24 Fig. Graphical representation of survival and hazard analysis in females representing IL8 expression in blood level.

S25 Fig. Graphical representation of survival and hazard analysis in females representing IL13 expression in tissue level.

S26 Fig. Graphical representation of survival and hazard analysis in females representing IL13 expression in blood level.

S27 Fig. Graphical representation of survival and hazard analysis in females representing IL12 expression in tissue level.

S28 Fig. Graphical representation of survival and hazard analysis in females representing IL12 expression in blood level.

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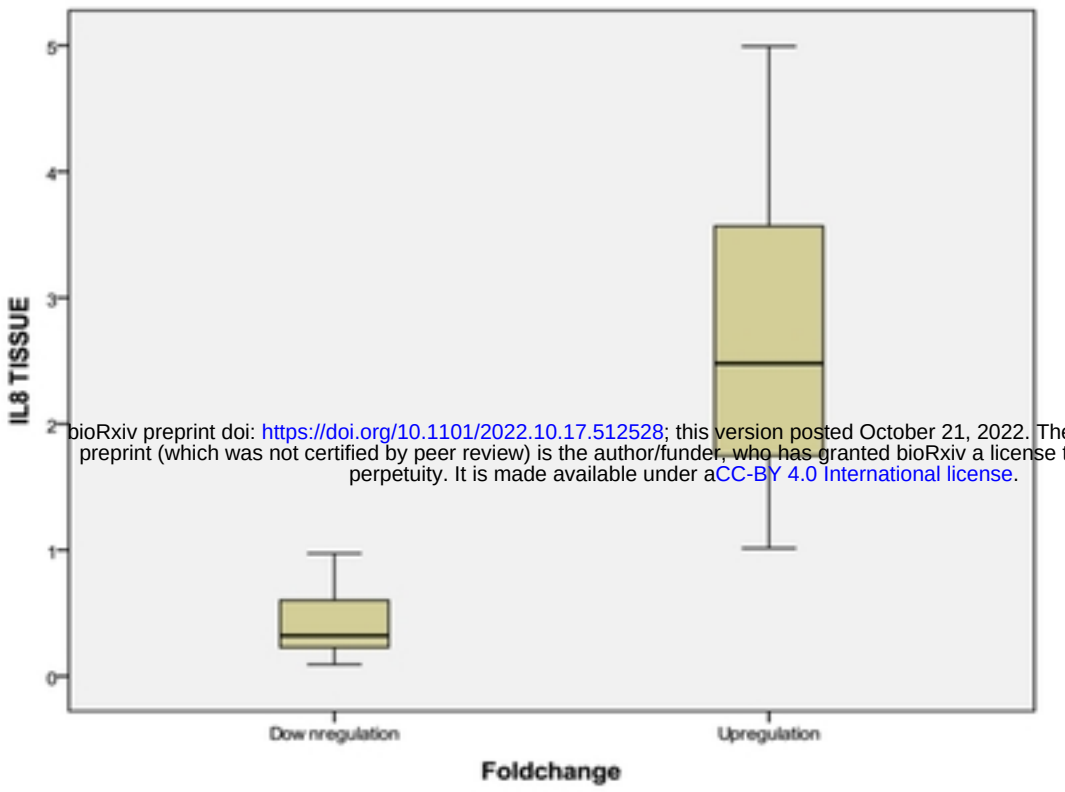

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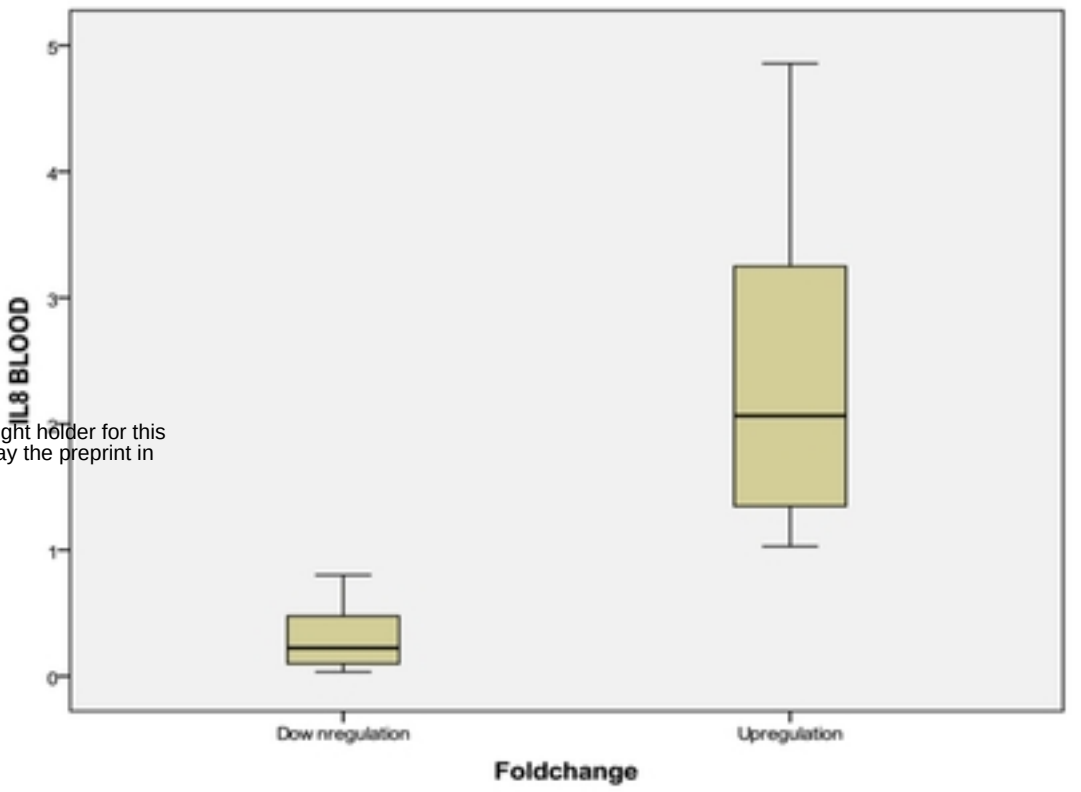

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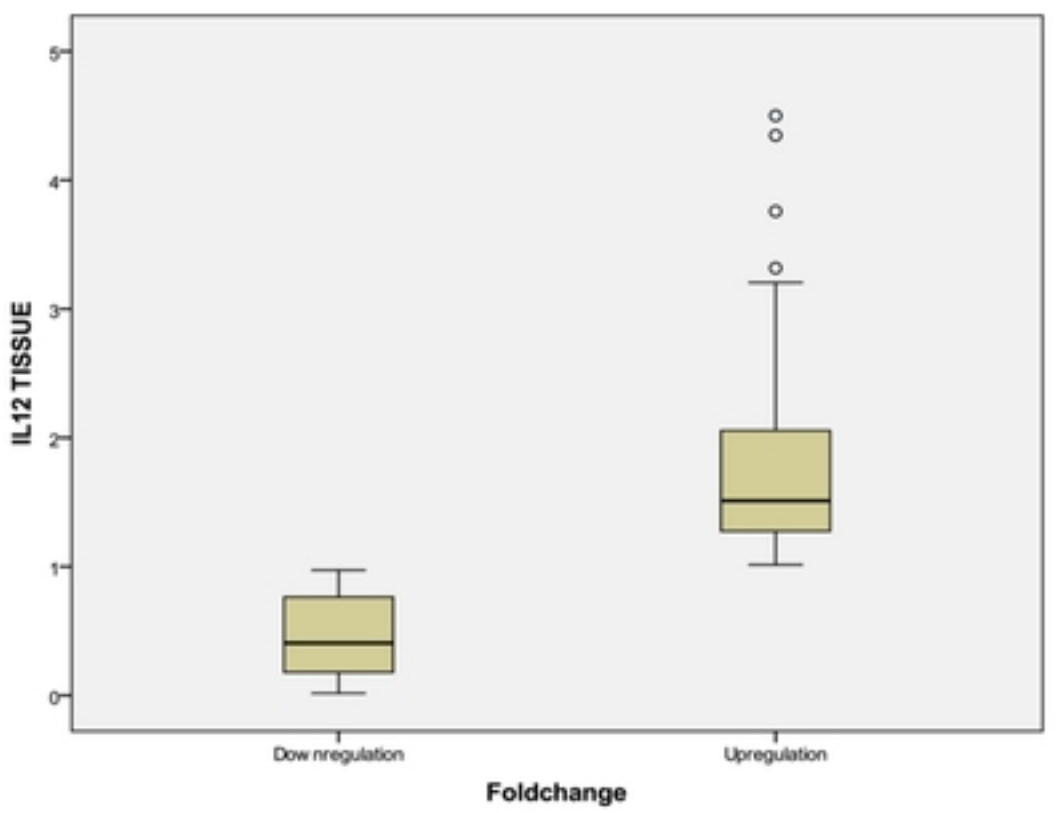

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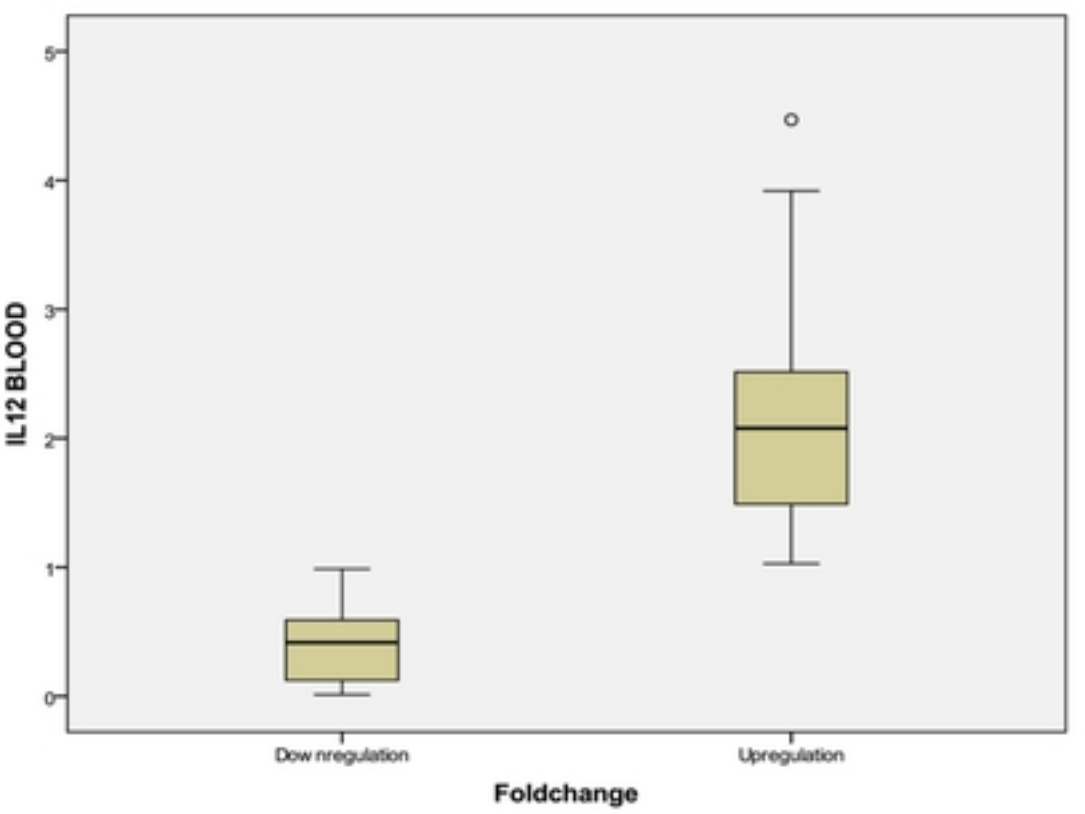

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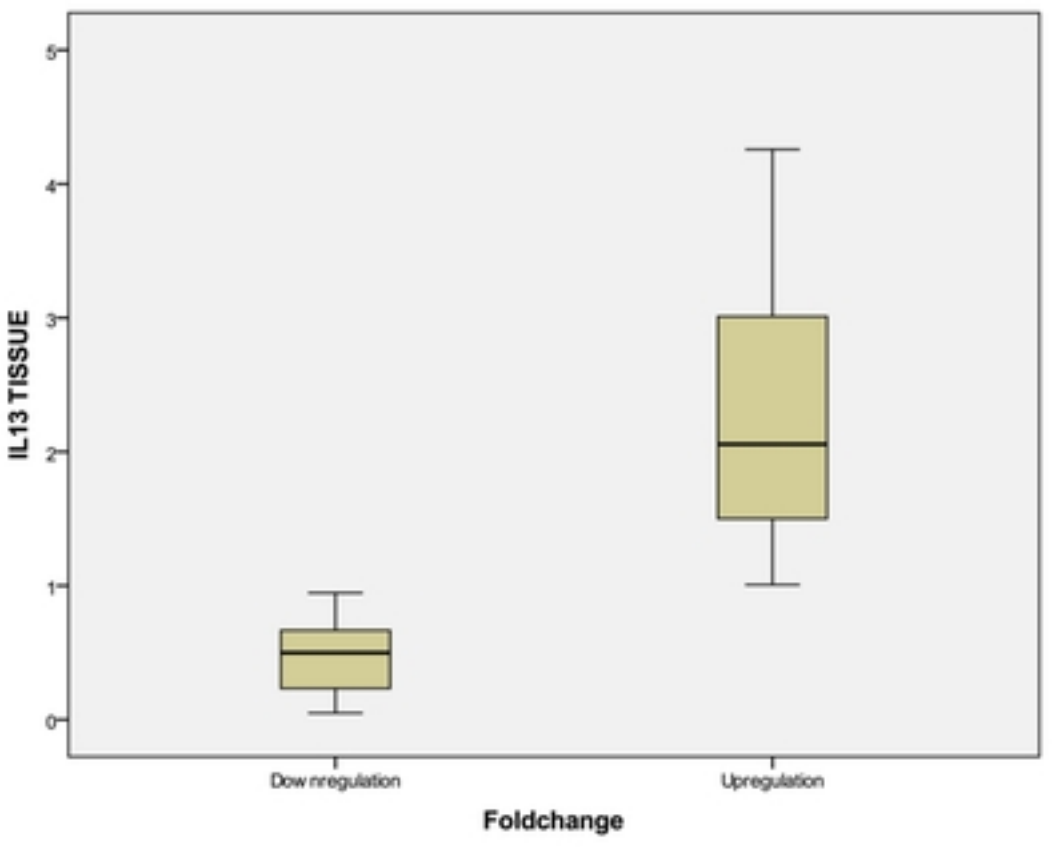

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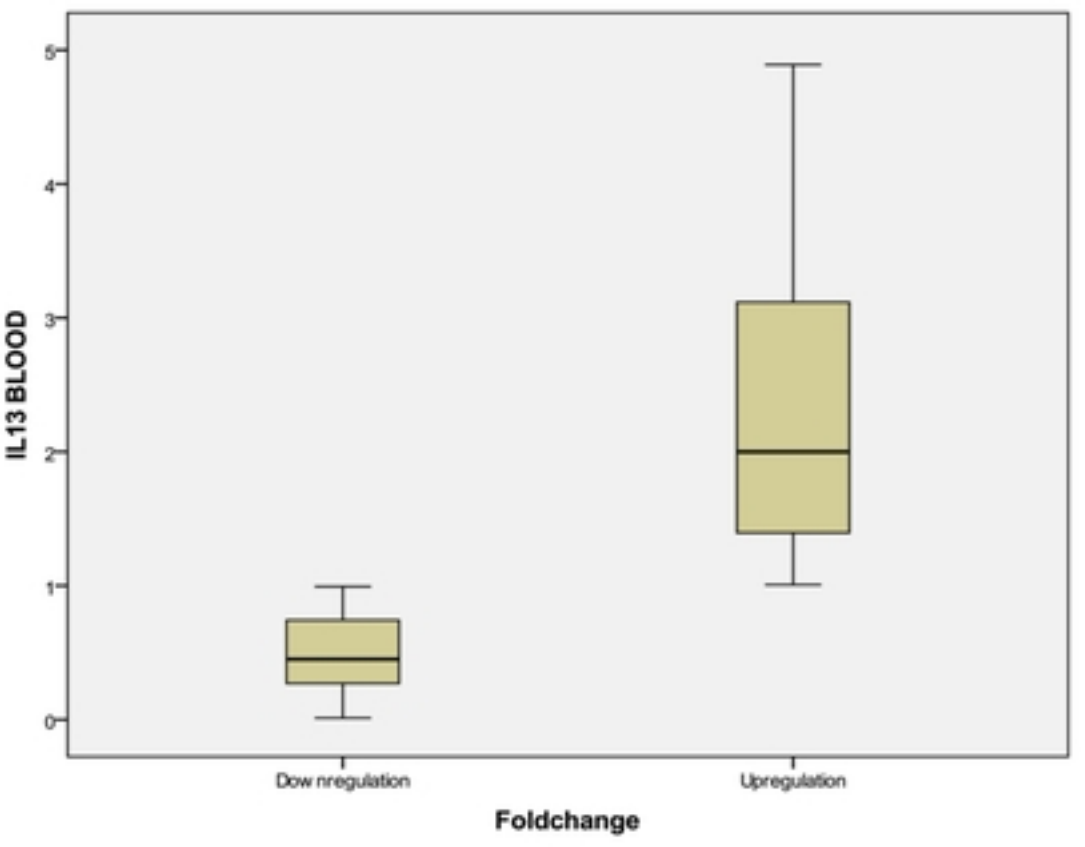
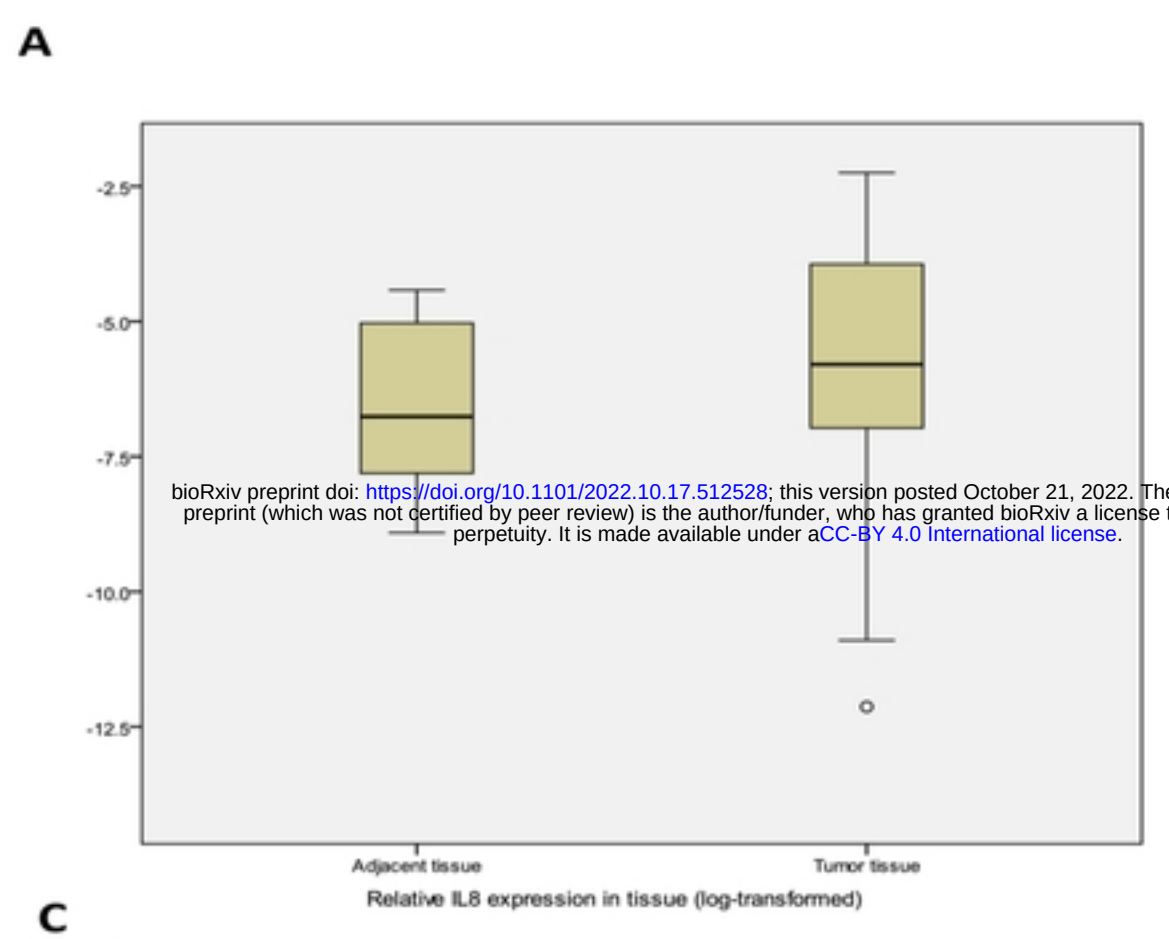
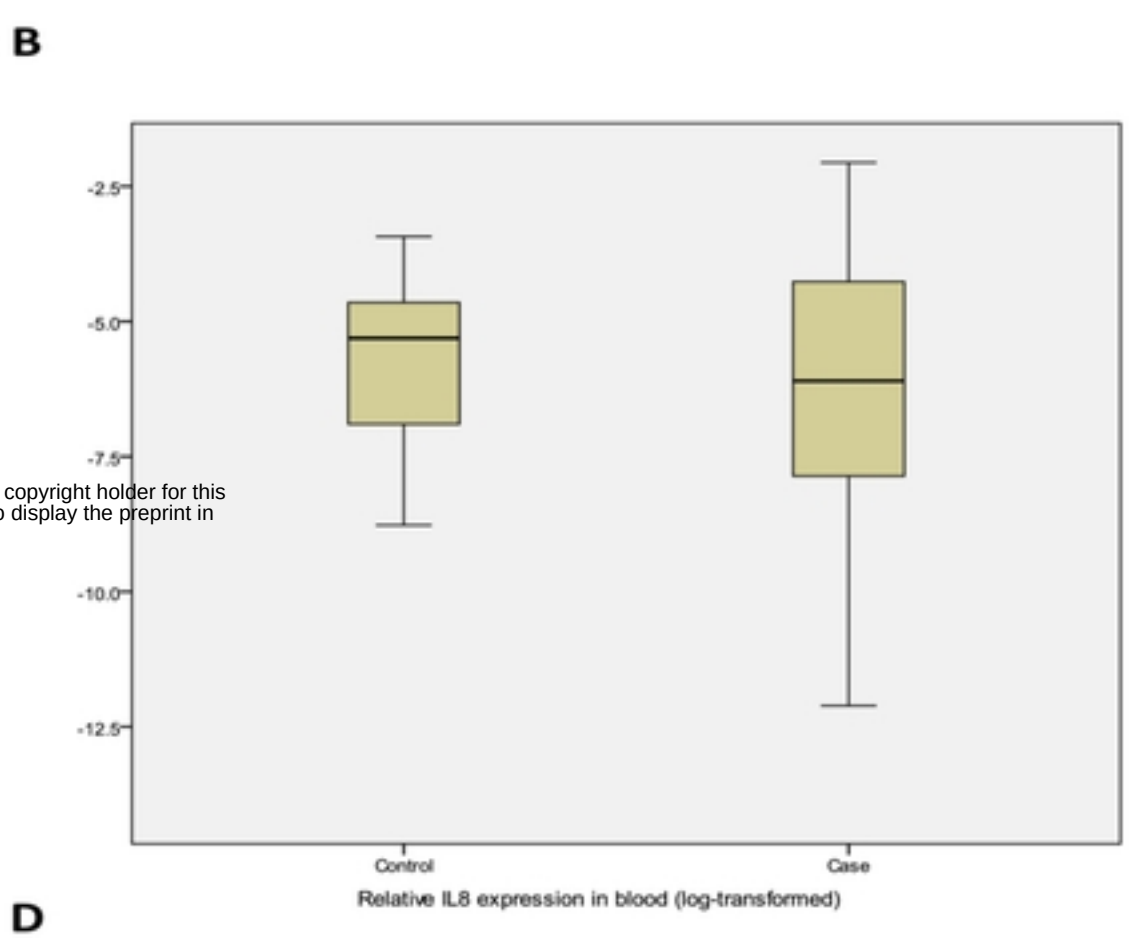
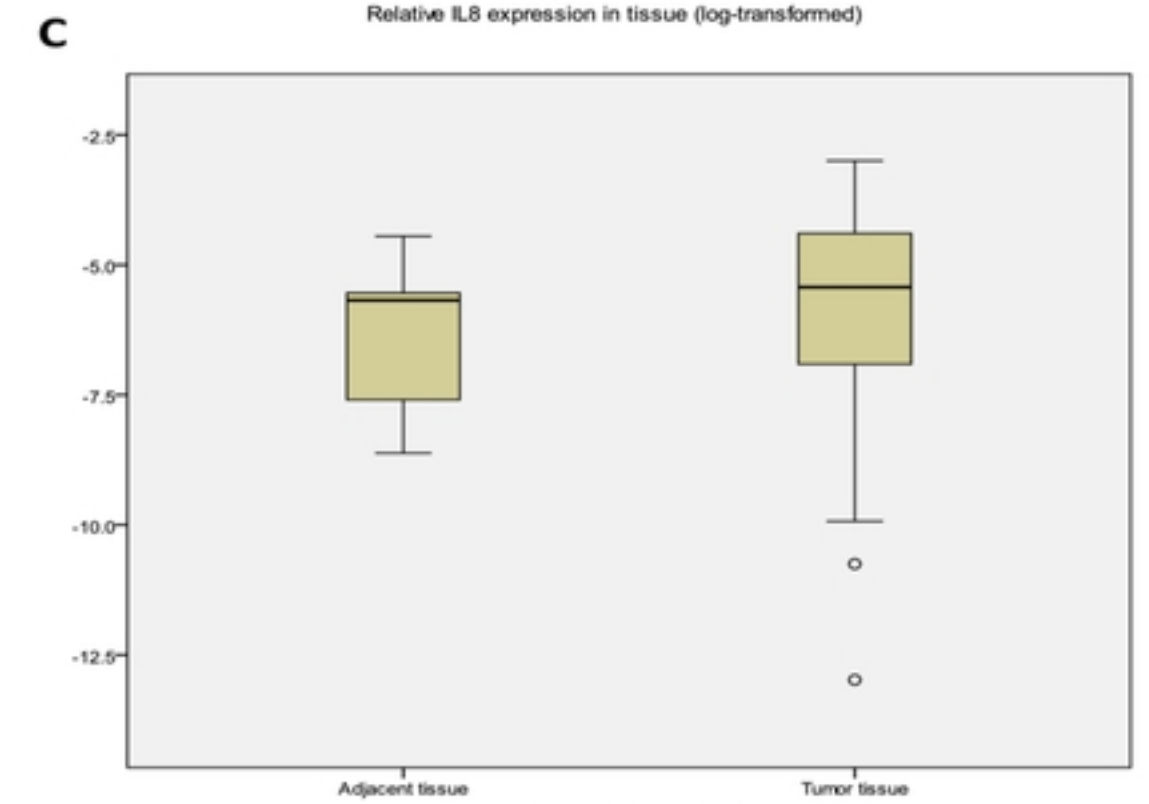
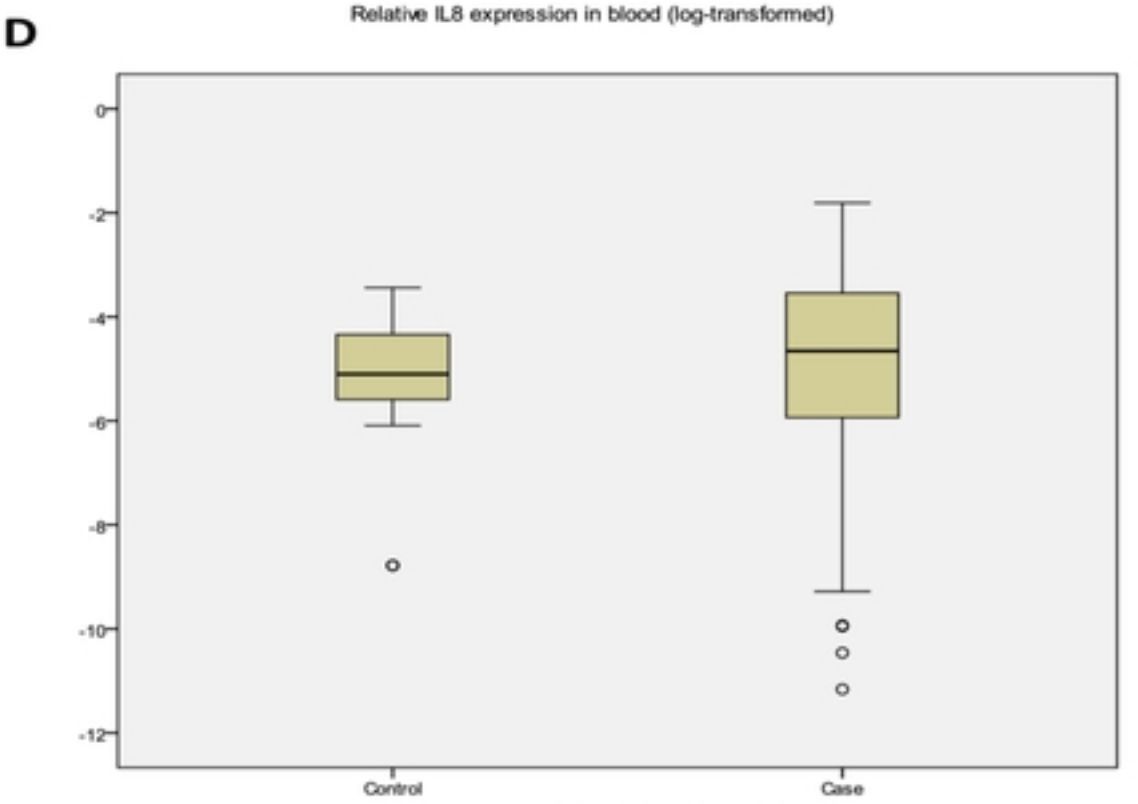


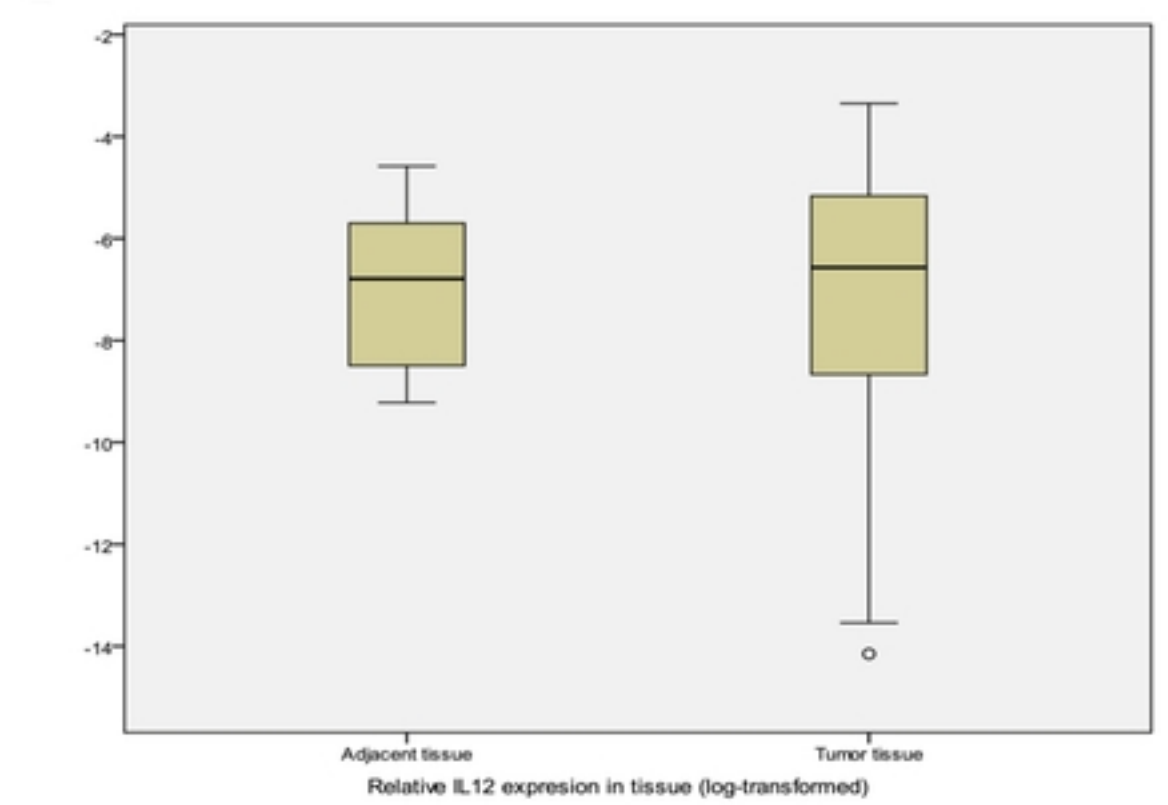
Fig 1











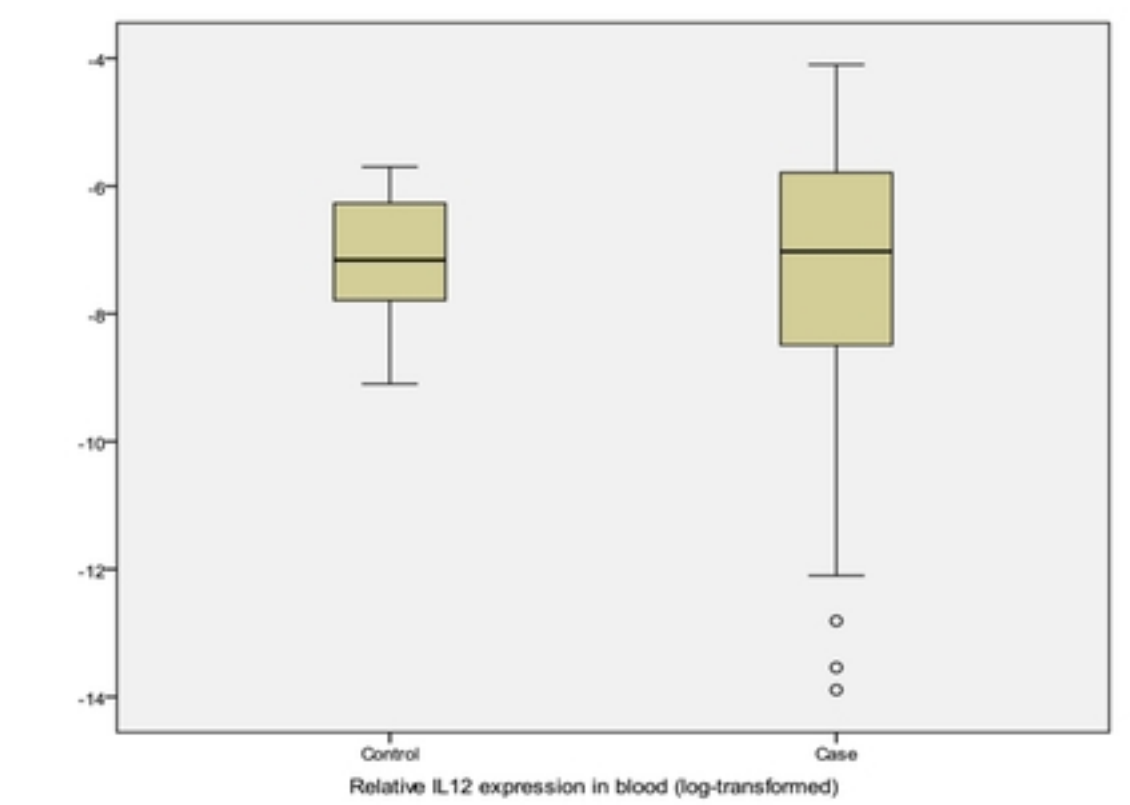


Fig 2