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Elevated cytokine expression in the orbitofrontal cortex of victims of suicide

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Abstract

Objective—Based on the reported association between cytokines with depression and suicide, and evidence of increased markers of inflammation in the brain of suicide victims, the present study examined the expression of cytokines in the orbitofrontal cortex of suicide victims.

Method—In a postmortem sample obtained from the Brodman area 11 of suicides (n = 34) and controls (n = 17), real-time RT-PCR was used to compare the expression of mRNA species for tumor necrosis factor-a (TNF-α), interleukin (IL)-1β, 4, 5, 6, and 13.

Results—Increased expression of IL-4 was found in women suicide victims and IL-13 in men suicide victims. Elevated but not significant cytokine expression was also observed for TNF-α in women suicide victims.

Conclusion—To our knowledge, these results provide the first evidence of the presence of mRNA transcripts of type 2 T-helper cytokines in the human orbitofrontal cortex and their increased expression in the brain of suicides.

Keywords

suicide; postmortem; gene expression; brain; neuroimmune

Significant outcomes

- Neuroimmune abnormalities in the brains of suicide completers are associated with type-2 (TH2) cytokines.
- These results are consistent with the reported association between allergies and suicide.

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Limitations

- Suicides and controls not matched for age.
- Limited information on psychiatric diagnoses and blood toxicology in the suicide group.

Introduction

Activation of the immune system by means of cytokine therapy is known to produce psychiatric side effects. The most common consequence of administering cytokines is the triggering of symptoms of depression (1–4). In addition, other serious mental complications are the occurrence of suicidal ideation, suicidal attempts, and completion (5–8). Case reports worldwide have documented death by suicide in patients receiving cytokines as immune boosting therapy to treat a variety of diseases including melanoma, hepatitis C, HIV infections, and multiple sclerosis (5–9). While there is a growing recognition of the participation of cytokines in the etiology of mood disorders, their role as pro-suicidal factors has been less studied (10,11).

In a recent study performed in postmortem brain tissue of patients that suffered from different psychiatric conditions including schizophrenia, major depression, and bipolar disorders, a positive correlation between markers of immune activation with suicide independent of the psychiatric disease was detected (12). This study reported that microglial cells in the frontal cortex, hippocampus, and dorsal thalamus of suicide victims had increased expression of markers of activation such as class II major histocompatible complex molecules. The regions of the brain examined have been reported to show neurochemical abnormalities in suicides and in depressed individuals and the authors suggested that some of these effects could be due to the actions of cytokines. However, data on cytokine expression in specific regions of the human brain are not yet available.

Based on the capacity of the brain to express mainly certain cytokines including tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-6 after activation of the immune system (13–15), the first objective of the present study was to compare the expression of these cytokines in the orbitofrontal cortical Brodman area 11 of suicides and controls. This area of the human cortex was selected based on its function in the neurobiology of suicidal behavior evidenced by postmortem and functional imaging studies (16–18). Moreover, in a recent epidemiological survey, we reported the association of suicide completion with increased aeroallergens during high atmospheric concentration of pollen (19). In addition, other epidemiological studies have reported the association of allergies with depression (20–22), and possibly suicide (23). Therefore, the second objective was to compare the expression of the major cytokines involved in allergies, including IL-4, IL-5, and IL-13.

Aims of the study

The aims of the study were to compare by real-time RT-PCR the expression of mRNA species for cytokines in postmortem brain samples of victims of suicide respect to controls that died of other causes.

Material and methods

Tissue collection and autopsy of brain samples

The human brain tissue was obtained from the Institute of Forensic Medicine of the Johann Wolfgang Goethe University in Frankfurt, Main, Germany. The research was approved by the

Institutional Review Board of the University. The basic characteristics of the subjects are shown in Table 1. Information on demographic data, cause of death and psychopathology of the decedents were derived from medical records, investigations of the coroner, the medical results of the examiner and interviews of next-of-kin. Postmortem tissue was derived from the orbitofrontal area (Brodmann area 11) of 34 suicides and 17 controls. Samples were selected by excluding those cases that showed signs of infarcts, subarachnoid hemorrhages or tumors. Almost all the subjects of our sample experienced brief deaths due to violent suicide methods or cardiac events with short states of agony (Table 1). All the subjects were Caucasian of German nationality.

RNA extraction and purification

Tissue pH is a robust indicator of RNA quality (24), and can affect mRNA determinations. The extraction of intact and biologically active mRNA after more than 4 days was reported by several groups (25–27). Therefore, we selected subjects with longer postmortem intervals (PMI) and focused on appropriate pH-values and stringent RNA quality control. As a result of this approach, pH values of suicides and controls were very similar (Table 1). The pH-value was measured by means of a pH-meter after homogenizing approximately 500 mg of brain tissue in 2 ml distilled water.

RNA was isolated from approximately 75 mg of frozen brain tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). The tissue samples were homogenized in 1 ml Qiazol Reagent using an Ultraturrax at 4°C for 20 s. The subsequent steps were carried out according to the manufacturers' protocol with an additional DNAse digestion to remove potential genomic DNA. The quality of the isolated RNA was first assessed by agarose gel electophoresis, and subsequently analyzed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All RNA samples showed clearly defined 28S and 18S ribosomal bands, indicating good RNA quality as proposed for quantitative PCR studies (28). The mean ribosomal RNA ratio (28S/18S), measured with the Agilent 2100 Bioanalyzer, was 1.60. The isolated RNA was stored at −80°C until further processing. Samples were shipped in dry ice to the University of Maryland within a 48 h window of time and were confirmed to be frozen at the time of arrival.

Real time RT-PCR

Five hundred nanograms of total RNA per sample were reverse transcribed into cDNA in a 20 μl reaction using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. This reagent contains a mixture of oligo (dT) and random hexamer primers that allow effcient reverse transcription of diverse RNA species. Real-time RT-PCR was conducted using the iQ SYBR Green Supermix (Bio-Rad) in a 50 μl reaction using the set of primers listed in Table 2. All sets of primers were tested in 1.8% agarose gel to confirm a single amplification product. The amplified products for cytokines were directly cloned into the pCRII-Topo vector (Invitrogen, Paisley, Scotland, UK) and sequenced to confirm their identity. All the primer pairs were designed using the Accelrys Gene 2.0v software (San Diego, CA, USA).

The real-time PCR reaction was run on a MyiQ instrument (Bio-Rad) with a three step cycling program as follows: an initial hot start for 5 min at 95°C followed by 40 cycles with a denaturation step of 15 s at 95°C, an annealing step of 30 s at 55°C, an extension step of 30 s at 72°C with the optics on at this last step. In preparation of a melt curve, the samples were heated for 1 min at 95°C then cooled for 1 min at 55°C, and the melt curve was executed in 10 s increments of 0.5°C with the temperature increasing from 55 to 95°C with the optics on. Effciency and consistency of the cDNA synthesis was determined by amplification of the human 18S gene as a control. For each round of amplifications, only those samples that were

within 1.6 cycles respect to the mean for 18S were considered for further analysis. This allows for comparisons of different initial amounts of total RNA within a range of 10-fold differences represented by the 3.3 cycles in real time determinations.

Data analysis for real-time RT-PCR

Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method established by Livak and Schmittgen (29) with the use of three control genes for normalization. Each cycle threshold of target gene was normalized respect to the averaged value obtained for 18S, gliceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin-β (ACTB) (Table 2). Relative expression was calculated by comparing normalized cycle threshold values respect to the mean value of controls. Each individual sample including those of the control cases was analyzed respect to this mean value of controls using the $2^{-\Delta\Delta Ct}$ algorithm giving a representative value of fold increase (higher than 1) or decrease (smaller than 1). Data is presented as mean ± standard error mean of fold increase respect to 1 (mean control value).

The following genes were analyzed according to their published accessible sequences of the Gene Bank: TNF-α (NM_000594); IL-1β (NM_000576); IL-6 (NM_000600); IL-4 (NM_000589); IL-5 (NM_00879); IL-13 (NM_002188), human ribosomal RNA subunit 18S (18S, K03432), GAPDH (NM_002046), ACTB (NM_001101).

Statistical analyses

Relative expression values of cytokines were logarithmically transformed to equalize standard deviations (SD) and produce normal distributions. Log transformed values for each cytokine were analyzed using the analysis of covariance (ANCOVA) (30). The ANCOVA model used consisted of cytokine expression values as dependent variable and suicide status, gender, age, and cause of death as independent variables. All tests were two-tailed with statistical significance set at alpha = 0.05. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Descriptive statistics of the sample

There were no differences between suicide victims and controls regarding the PMI gender or pH. However, the suicide victim group was slightly younger (52 years) ($n = 34$) than the control group (61 years) ($n = 17$). In suicide victims, data for blood alcohol (mg alcohol/g blood) was available for 82% of the cases ($n = 28$) whereas for the control group, data was only available for 17% of the cases ($n = 3$). Suicide victims tested positive for alcohol in 41% ($n = 14$) of the cases and negative in 38% of the cases ($n = 13$). Controls tested positive for alcohol in three cases from 17 and no information was available in the rest of the cases. Suicide victims included nine cases of major depression and one case of bipolar I disorder. Two cases had a history of alcohol abuse (AA). Psychological information was not available for the rest of the cases.

Identification of mRNA species for cytokines in brain tissue

Specific products using the real-time RT-PCR amplification conditions described were detected for all the set of primers. Figure 1 shows a 1.8% agarose gel electrophoresis of final products after 40 cycles of real-time PCR reaction. Single bands corresponding to specific cytokines can be observed on top of the band corresponding to the primers. The image also shows that when the reverse transcriptase enzyme step was omitted (RT−), no product was detected ruling out potential genomic contamination and confirming that the product originated from an RNA specie. The only exception to this rule was the band corresponding to IL-5. Due to the size of the amplified product (62 base pairs, Table 2), the product merged with the band

of the primers. We confirmed amplification of this product by cloning and sequencing as described in methods.

Specific products were detected for all the cytokines and for all the cases analyzed. Melting curves showed single peaks in all the cases.

Cytokine expression in Brodman area 11

Analysis of the real-time data obtained for cytokines showed that the ratio of mRNA expression of cytokines to that of the housekeeping genes was constant between controls and suicide victims indicating that the data was suitable for further analysis. Using the averaged value for the three control housekeeping genes the ratios of expression for cytokines considering controls and suicides were as follows: 2.12 ± 0.01 for IL-13; 2.25 ± 0.01 for IL-1; 2.26 ± 0.01 for TNF-α; 2.27 ± 0.02 for IL-6 and 2.3 ± 0.01 for IL-4 and IL-5.

Analysis of covariance for the classical brain cytokines, IL-1β, IL-6, and TNF-α showed no significant difference between controls and suicides and no effect was detected for gender (Fig. 2). On the other hand, the ANCOVA revealed significant differences for IL-4 between suicide victims and controls ($F = 6.51$; $P = 0.0136$) and between women and men ($F = 30.3$; $P < 0.0001$). The estimated ratio of geometric means of suicide victims vs. control was 1.764 and the ratio of men vs. women was 0.326 with increased values in women victims of suicide (Fig. 3). Similarly, a significant difference between suicides and controls were observed for IL-13 ($F = 14$; $P = 0.0004$) (Fig. 3) and between women and men ($F = 4.38$; $P = 0.041$). The estimated ratio of geometric means of suicide vs. control was 2.240 and the ratio of men vs. women was 1.512. No significant differences were found for IL-5. No effects were detected for any cytokine when considering age and cause of death.

Further analyses of individual cases revealed that in women victims of suicide, ten cases of 14 showed increased values compared to control for IL-4 (Table 3) with six of them having also increased values of IL-6. Moreover, in men 13 cases of 20 showed increased values for IL-13 with seven of them also having elevated TNF-α values. However, no significant correlations among cytokines were detected. The number of suicide cases showing increased, no change or decreased values respect to controls for all the cytokines are shown in Table 3.

Discussion

The present study is the first to document and compare the presence and relative expression of mRNA species for cytokines in human cortex in psychiatric non-neurological cases. Moreover, the present study reports for the first time increased levels of type 2 T-helper cytokines in suicide victims. These results provide additional evidence of the potential participation of inflammatory processes in the brain, in particular cytokines, in suicide.

The orbitofrontal cortex including Brodman area 11 has been strongly implicated as a structure associated with inhibition of aggression, impulsivity and suicidal behavior (16,31,32). In addition, postmortem studies have demonstrated neurotransmitter abnormalities in this area of the brain of suicides such as decreased serotonin transporter binding sites and increased 5-HT1A and 5-HT2A receptor binding (32,33). These findings are also consistent with functional imaging studies that demonstrate decreased activity in the ventral prefrontal cortex associated with suicidal and aggressive behaviors (17,31,34). In sum, there is strong evidence from functional imaging and postmortem studies implicating the orbitofrontal cortex, including Brodman area 11, in inhibiting or modulating many pro-suicidal factors including hopelessness, impulsivity, aggression, and anxiety. Therefore, the presence of mRNA for cytokines in this region of the human cortex may be of relevance because it indicates local

production of molecules with potential to influence important brain functions related to suicidal behavior.

Cytokines are regulatory peptides that participate in host defense and repair processes of tissues. In the brain, the most studied cytokines are IL-1β, TNF-α, and IL-6 and have been implicated in a variety of normal and pathological conditions (2). Among many roles, they modulate neuroendocrine functions, sleep, sickness behavior and participate in neuroinflammatory and neurodegenerative processes (2). They have also been implicated in the neurobiology of mood disorders (1–4,13,14). Data on the functions of IL-4, IL-5, and IL-13 in the brain is less available. It has been reported that they counter-balance pro-inflammatory processes with potential beneficial effects in experimental models of autoimmune encephalomyelitis (35). However, studies using cultured microglial cells have shown effects of activation and production of inflammatory mediators in response to these cytokines (36–38). In addition, there is compelling evidence indicating that these cytokines may be involved in the neuropathology of schizophrenia (39,40). The present study showing increased levels of IL-4 and IL-13 transcription in the orbitofrontal cortex of suicides suggests that these cytokines may affect neurobehavioral processes relevant to suicide. Nevertheless, the present study cannot address if the expression of cytokines is secondary to presuicidal anguish or if it is a factor of vulnerability predisposing individuals to suicide.

Several mechanisms have been proposed to explain how cytokines may affect brain function and behavior. Among them, the most studied are interactions with the hypothalamic–pituitary–adrenal (HPA) axis and with the enzyme indoleamine-2,3-dioxygenase (IDO). In the first case, cytokines can induce activation of the HPA-axis resulting in altered cortisol levels with detrimental effects for neurons. The latter relates to the activation or inhibition of the IDO enzyme that will ultimately result in altered serotonin metabolism and production of neuroactive substances. For reviews on these issues see (39–42).

It has been reported that allergies which involve the production of IL-4 and IL-13 are associated with increased suicide rates in women (19,23). The present findings for IL-4 in women suicide victims are consistent with these epidemiological studies. Moreover, elevated IL-13 in suicide women was observed but the differences were not significant. The finding of increased IL-13 in suicide men may be related to several other factors including those related to the reported gender effects in suicidal behavior (43,44) and the specific functions of this cytokine (45). In this regard, it should be highlighted that while there is abundant information about gender differences in suicide risk factors and suicidal behavior, to our knowledge, postmortem studies have not reported major neurochemical or gene expression differences between the brain of suicides women and men. The observed sex-differences in cytokine expression in the present study may be of relevance because sex-differences in the immune and cytokine response of the brain have been reported in experimental animal models (46–48). Therefore, cytokines may further our understanding of neurochemical sex-differences in the brain that may be related to sex-differences in behavior.

The present study contains several limitations that should be considered when interpreting the current results. The most important are non-matched controls for age, incomplete toxicology (82% of cases), and limited information on psychological diagnosis (26%). In the first case, a correlation between cytokine expression and age was not detected, a finding consistent with the study of Steiner et al. (12). However, for the cases of alcohol and suffering from a psychiatric condition, the present study may not represent accurate interactions of these factors with the expression of cytokines. This limitation is relevant considering the reported effects of alcohol on inflammatory processes in the brain (49,50) and the association between cytokines and psychiatric diseases (40,41). Future studies matching controls and suicides for these factors, expanding the analysis to other brain regions, and including additional cytokines will

be necessary to strengthen the evidence on the involvement of cytokines in suicide. If so, studies aimed at determining the cellular origin of mRNA species for cytokines in the brain may become of relevance.

If further corroborated in larger samples, the present results may contribute to identify previously unexplored factors that may participate in the pathophysiology of suicide.

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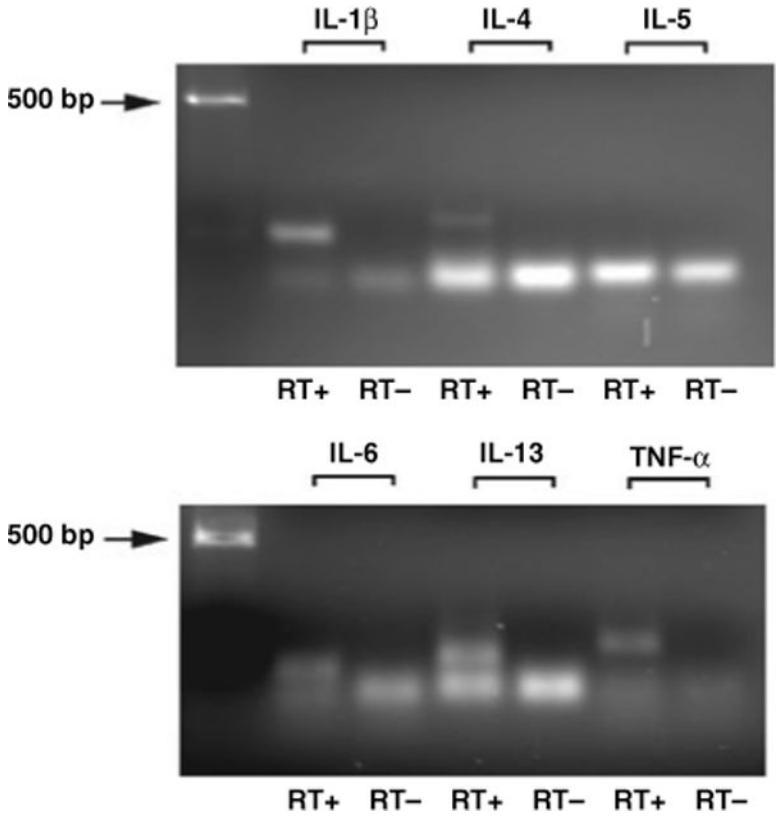

Fig. 1.

Agarose gel electrophoresis images of amplified products after 40 cycles of real-time RT-PCR reaction using the set of primers listed in Table 2. Single bands corresponding to specific cytokines can be observed on top of the primer-dimer bands. RT+: reaction containing cDNA originated from total RNA. RT−: no amplification of product when RNA was amplified without reverse transcriptase step.

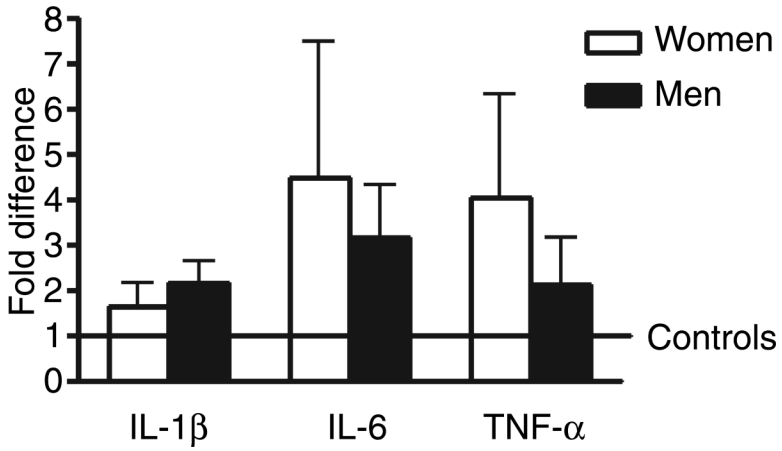

Fig. 2.

Relative mRNA expression levels of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) in Brodman area 11 of suicide victims with respect to controls that died of other causes. Values are fold increase ±SEM respect to the mean of controls (represented by the number 1).

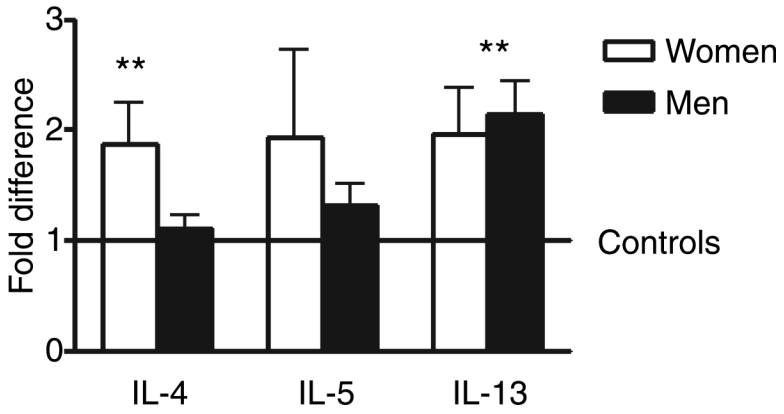

Fig. 3.

Relative mRNA expression levels of interleukin-4 (IL-4), IL-5, and interleukin-13 (IL-13) in Brodman area 11 of suicide victims with respect to controls that died of other causes. Values are fold increase ±SEM respect to the mean of controls (represented by the number 1) (**$P <$ 0.001 respect to control).

Table 1

Demographic and tissue characteristics of suicides and controls subjects. Mean ± standard deviation (SD) values for each group are provided for age, postmortem interval (PM h) and pH

Group	Sex	Age	Cause of death	Diagnosis (DSM-IV)	BAL	PM h	pH
Suicide	F	42	Intoxication		Neg.	11	6.70
Suicide	F	49	Hanging	MDD	0.6	72	6.75
Suicide	F	38	Hanging		Neg.	66	6.80
Suicide	F	61	Hanging		1.56	91	6.30
Suicide	F	83	Hanging		Neg.	48	6.50
Suicide	F	63	Jumping from height	MDD		52	6.65
Suicide	F	39	Jumping from height		1.91	11	6.85
Suicide	F	67	Jumping from height		Neg.	115	6.35
Suicide	F	57	Intoxication	MDD	Neg.	110	6.30
Suicide	F	81	Cutting	MDD	Neg.	69	6.65
Suicide	F	31	Intoxication	BD I		72	7.00
Suicide	F	65	Intoxication			48	6.60
Suicide	F	46	Intoxication		1.27	39	6.65
Suicide	F	53	Jumping from height	MDD	0.002	4	6.75
n = 14 (Mean ± SD)		55.3 ± 15.7				57.7 ± 34.5	6.6 ± 0.2
Suicide	M	33	Gunshot		2.24	24	6.90
Suicide	M	59	Hanging	MDD		72	6.90
Suicide	M	33	Hanging		Neg.	24	6.70
Suicide	M	51	Hanging		0.19	115	6.80
Suicide	M	72	Jumping from height		Neg.	86	6.85
Suicide	M	55	Hanging		Neg.	88	6.75
Suicide	M	57	Hanging		1.7	97	6.70
Suicide	M	66	Hanging	MDD	Neg.	48	6.85
Suicide	M	72	Hanging		1.59	92	6.70
Suicide	M	29	Hanging		0.96	32	6.60
Suicide	M	27	Hanging		0.29	115	6.90
Suicide	M	38	Jumping from height	MDD	Neg.	48	6.90
Suicide	M	60	Hanging	AA	0.04	96	6.70
Suicide	M	48	Hanging			48	6.60
Suicide	M	66	Hanging		Neg.	110	6.70
Suicide	M	57	Intoxication	MDD		72	6.65
Suicide	M	43	Gunshot	AA	2.92	100	7.10
Suicide	M	64	Cutting		Neg.	13	6.75
Suicide	M	42	Hanging		0.001	40	7.05
Suicide	M	33	Suffocation		Neg.	31	6.7
n = 20 (Mean ± SD)		50.2 ± 14.6				67.5 ± 32.4	6.8 ± 0.1
Control	F	67	Heart attack			72	6.40
Control	F	71	Pancreatitis			48	6.55
Control	F	76	Heart attack			115	6.60
Control	F	53	Heart attack			67	6.75
Control	F	62	Heart attack			115	6.80
n = 5 (Mean ± SD)		65.8 ± 8.8				83 ± 30.2	6.64 ± 0.1
Control	M	63	Emphysema			98	6.95
Control	M	59	Heart attack			48	6.80
Control	M	65	Heart attack			87	7.00
Control	M	77	Aneurysm			76	6.80
Control	M	62	Accident		1.68	15	6.70
Control	M	69	Heart attack		0.23	110	6.80
Control	M	54	Heart attack			46	6.80
Control	M	37	Heart attack			61	6.65
Control	M	58	Heart attack			96	6.65
Control	M	48	Heart attack			48	6.95

Group	Sex	Age	Cause of death	Diagnosis (DSM-IV)	BAL	PM h	pH
Control	M	54	Heart attack		0.41	48	6.30
Control	M	55	Heart attack			70	6.45
n = 12 (Mean ± SD)		58.4 ± 10.2				66.92 ± 27.6	6.7 ± 0.2
Total suicides (*n* = 34), mean ± SD		52.3 ± 15				63.5 ± 33	6.7 ± 0.2
Total controls (*n* = 17)		60.6 ± 10				71.7 ± 28	6.7 ± 0.19

BAL, blood alcohol level; PM h, postmortem interval; AA, alcohol abuse; BD I, bipolar I disorder; MDD, major depressive disorder.

Table 2

Primer sequences used in realtime RT-PCR determinations

Gene	GeneBank		Primer sequence	Region	Product (bp)
TNF-α	NM_000594	Fwd	AAGCAACAAGACCACCACTTCG	1010−1157	148
		Rev	TCTCCAGATTCCAGATGTCAGGG		
IL1-β	NM_000576	Fwd	GCACCTTCTTTCCCTTCATCTTTG	359−486	128
		Rev	GCTTTTTTGCTGTGAGTCCCG		
IL-6	NM_000600	Fwd	GAGAAGATTCCAAAGATGTAGCCG	160−255	96
		Rev	AGATGCCGTCGAGGATGTACC		
IL-4	NM_000589	Fwd	AAGCAAAAAGCCAGCAGCAGCC	10−94	85
		Rev	ACAAAGTTTCAGCATAGGAAATTAC		
IL-5	NM_00879	Fwd	GCATTGGTGAAAGAGACCTTGG	126−187	62
		Rev	TCATTGGCTATCAGCAGAGTTCG		
IL-13	NM_002188	Fwd	ACCCACTTCACACACAGGCAAC	794−918	124
		Rev	ACAGTCTTCCCCAATCCCCAAC		
18s	K03432	Fwd	CCGATAACGAACGAGACTCTGG	1514−1606	93
		Rev	TGAACGCCACTTGTCCCTCTAAG		
GAPDH	NM_002046	Fwd	TTCGTCATGGGTGTGAACC	493−631	139
		Rev	TGGTCATGAGTCCTTCCAC		
ACTB	NM_001101	Fwd	CCACGAAACTACCTTCAACTC	895−1027	133
		Rev	AGTGATCTCCTTCTGCATCC		

TNF-α, tumor necrosis factor alpha; IL, interleukin; GAPDH, gliceraldehyde-3-phosphate dehydrogenase; ACTB, actin-β.

Table 3

Total number of suicide victims showing increased, no change or decreased expression respect to controls for all the cytokines analyzed

Suicides	IL-1β	IL-6	TNF-α	IL-4	IL-5	IL-13
Women (*n* =14)						
Increased	7	10	7	10	5	8
No change	3	0	2	3	6	4
Decreased	4	4	5	1	3	2
Men (*n* = 20)						
Increased	12	9	10	7	7	13
No change	3	2	3	4	7	4
Decreased	5	9	7	7	6	3

TNF-α, tumor necrosis factor alpha; IL, interleukin.