

Cytokine profiles in patients receiving antioxidant therapy within the ANTICIPATE trial

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Abstract

AIM: To measure a broad profile of pro- and anti-inflammatory cytokines in patients with clinically proven chronic pancreatitis (CP) taking either antioxidant therapy or placebo as part of the larger ANTICIPATE study.

METHODS: Patients with chronic pancreatitis were recruited to the ANTICIPATE study following informed consent and were randomised to intervention with either antox version 1.2-based antioxidant therapy or placebo. After a separate ethics committee amendment a subgroup of 7 patients from either arm of the study were selected for additional analysis of cytokines. Cytokines were measured at baseline and after 6 mo of either antox therapy or placebo by biochip array and enzyme-linked immunosorbent assay.

RESULTS: Antioxidant therapy and placebo groups were well-matched in terms of age, gender, aetiology of CP, opiate use and disease duration. Baseline antioxidant levels were similar in patients allocated to the antioxidant group as compared to the group allocated to placebo. After 6 mo of antioxidant therapy

there was significant elevation in vitamin C levels in the intervention group: 17.6 µg/mL (12.8-29.3 µg/mL) compared to 4.8 µg/mL (1.6-9.1 µg/mL) in placebo ($P < 0.001$; 95%CI: 9.0-20.2) with similar trends in selenium levels. There was no elevation in a broad array of pro- and anti-inflammatory cytokines in the antioxidant group compared to placebo [interleukin (IL)-1B, IL-4, IL-6, IL-10, tumor necrosis factor- α] either at baseline or after 6 mo of antioxidant therapy.

CONCLUSION: Cytokine levels were low at baseline and at 6 mo despite a significant elevation in plasma antioxidants. In patients with CP, with opiate-dependent abdominal pain, circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of inflammation.

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Key words: Chronic pancreatitis; Antioxidant therapy; Cytokine

Core tip: This study examines cytokine levels in a subset of patients recruited from within the ANTICIPATE randomized controlled trial of antox for painful chronic pancreatitis. At baseline, pro- and anti-inflammatory cytokine levels were within the laboratory reference range in patients allocated to the antioxidant arm and those allocated to placebo. After 6 mo of intervention with antox, there was a significant elevation in antioxidant levels in patients in the active treatment arm. This was not associated with any change in either pro- or anti-inflammatory cytokine levels. In patients with chronic pancreatitis, with opiate-dependent abdominal pain, circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of inflammation.

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INTRODUCTION

The oxidative stress hypothesis proposed that cell injury in chronic pancreatitis (CP) was mediated at the acinar level by short-lived oxygen free radicals produced as a result of imbalance in the physiological processes producing these agents and those pathways involved in deactivating them^[1]. A key component of this theory was that the methionine transsulfuration pathway which yields glutathione (important in the quenching of antioxidants) is overwhelmed in patients with CP as the detoxification of xenobiotics by cytochrome P450 led to overproduction of oxygen-derived free radicals^[1]. There was evidence that the dietary intake of some patients with CP was deficient in selenium, methionine and vitamin C, key cofactors in these transsulfuration pathways^[2]. This finding was supported by evidence showing that plasma/blood levels of circulating antioxidants were low in CP compared to control^[3]. The logical completion of this paradigm was the development of antioxidant therapy - a pharmacological preparation containing methionine, vitamin C, vitamin E and selenium and designed to restore these critical co-factors to patients with CP^[1]. Early clinical trials of antioxidant therapy failed to establish evidence of clinical efficacy and thus the treatment was not widely accepted. To address this issue, we conducted and reported the largest randomized controlled trial of antox for treatment of pain in chronic pancreatitis - the ANTICIPATE study^[4]. In this, 356 patients with CP were screened for eligibility, 92 randomised and 70 completed intervention with 6 mo of antioxidant therapy or matched placebo. At the end of this period there was no difference between treatment and placebo in the primary endpoint of abdominal pain as assessed by a numerical rating scale or in secondary endpoints of pain assessed by pain diaries and quality of life assessed by validated questionnaire^[4]. However, blood and plasma antioxidant levels were significantly elevated in patients in the treatment group^[4]. In keeping with other clinical studies of antioxidant therapy in chronic pancreatitis with clinical endpoints there is little information on the effects of intervention on inflammatory markers.

The present study does provide unique data on cytokine profiles in patients with chronic advanced pancreatitis at their end disease stage receiving antioxidant therapy and in a matched cohort receiving placebo and provides negative results which should be regarded as important pilot data. Thus, although the principal findings were negative, the ANTICIPATE study provided a unique vehicle with which to assess the potential interaction between antioxidant therapy and cytokine markers of inflammation and fibrosis in chronic pancreatitis. To the best of our knowledge, this interaction has never previously been studied.

In chronic pancreatitis there is evidence that levels

of platelet-derived growth factor-BB and transforming growth factor (TGF)- β 1 are elevated and that these cytokines play an important role in pancreatic fibrosis^[5]. Pancreatic stellate cells are activated by alcohol in CP and are key mediators of subsequent inflammatory changes and fibrosis with these changes being modulated by cytokines including epidermal growth factor^[6,7]. Pancreatic ductal epithelium produces TGF- β which also mediates fibrosis^[8]. Thus cytokines are known to be key mediators of inflammatory and fibrotic change in CP.

The aim of the present study was to examine circulating cytokine levels in a cohort of patients within the ANTICIPATE study. The principal endpoint was to assess whether there were differences between patients receiving antioxidant therapy and those receiving matched placebo.

MATERIALS AND METHODS

Study design

This is a case-control analysis of a sub-group of patients recruited from both arms of the ANTICIPATE double-blind, placebo-controlled, randomised trial of Antox version 1.2 (Pharma Nord, Morpeth, United Kingdom) in patients with painful chronic pancreatitis^[4].

Setting

Tertiary care academic medical centre was eventually chosen as setting in which to implement the requirement.

Inclusion/exclusion criteria

The inclusion criteria were as for the main ANTICIPATE study and can be summarised as follows: patients with evidence of chronic pancreatitis on cross-sectional imaging together with evidence of impairment of pancreatic exocrine function as assessed by assay of faecal elastase. Patients who did not meet these criteria were excluded as were patients with evidence of malignancy. The inclusion/criteria for the main study are provided in detail elsewhere^[4].

Identification and selection of study sub-group

Recruitment to ANTICIPATE commenced in February 2008 and a protocol amendment to permit additional enrolment to the present study was approved 6 mo later. Patients recruited to ANTICIPATE were allocated to receive either 6 mo intervention with antox or matched placebo in a randomised, double-blind, placebo-controlled fashion. Those patients selected to participate in this study signed an additional consent form. No additional inclusion or exclusion criteria were used. Allocation arm was unknown during the conduct of ANTICIPATE and patients were stratified at enrolment to this study by whether or not they had undergone prior pancreatic intervention (either surgical or endoscopic). Blood samples were drawn from 22 patients in the "prior intervention" stratification arm and from 15 in the "no prior intervention" stratification arm. Following the code break at the end of the clinical ANTICIPATE study, investigators were notified which patients had been allocated to active drug and which had

been allocated to placebo. At this point, a study population of 10 consecutive patients from each arm of the study was identified (total 20 patients). Allowing for loss to follow-up in 6 patients in whom blood samples for cytokine analysis were not taken after the original baseline assays a final study population of 7 patients treated with antox for 6 mo and 7 patients treated with placebo was obtained.

Assays

Full blood count (haemoglobin and white cell count) was measured at baseline and at 2, 4 and 6 mo. C-reactive protein (CRP) levels were also measured at these time points. Antioxidant levels comprising the following: selenium, vitamins C and E, β -carotene and glutathione were measured at baseline, study mid-point and at 6 mo. A range of cytokines were measured at baseline and at 6 mo as follows: pro-inflammatory cytokines interleukin (IL)-1 β , IL-6, tumor necrosis factor alpha; anti-inflammatory cytokines: IL-4, IL-10; chemokines: IL-8, IL-18, monocyte chemoattractant protein 1; the T cell regulatory cytokine IL-2; the angiogenic signalling protein vascular endothelial growth factor (VEGF) and epidermal growth factor an important regulator of cellular proliferation, differentiation and survival^[7].

Methods of measurement

Full blood count was measured by the haematology department of the Manchester Royal Infirmary with CRP being measured in the clinical biochemistry service and these results were available to clinicians to guide on-going management during the study. Antioxidant levels were measured by the pancreatic laboratory of the Manchester Royal Infirmary. The results of these assays were available during the study. Cytokine assays were undertaken by Bio-chip Arrays and enzyme-linked immunosorbent assay by Randox laboratories, Crumlin, Northern Ireland. These were analysed as a batch at the end of the study.

Sample collection

Non haemolysed and non-lipaeic serum and plasma were used for the Biochip array. Samples were collected into leak-proof, non-absorbent plastic containers. After collection, samples were aliquoted into containers and stored at -70 °C. Repeated freeze/thaw cycles were avoided. Samples were labelled prior to transportation on dry ice to Randox laboratory, Crumlin, Northern Ireland *via* a secure, approved courier.

Interference

The effect of bilirubin, haemoglobin, triglycerides and lipids were assessed to establish the level at which the interference caused a significant increase or decrease in assay performance. The criterion set for this was that analyte recovery (all cytokines) should not vary from base recovery by more than 10%.

Ethics committee approvals

The original full study protocol was approved by the

North West Regional Ethics Committee (MREC, 07/MRE08/13) and the United Kingdom Medicines and Health products Regulatory Agency (MHRA, 2006-006958-10). This cytokine subgroup study was approved by the North West Regional Ethics Committee as a separate amendment. The master study ANTICIPATE from within which these patients were recruited was registered with the International Registry of Randomized Controlled trials and allocated the number ISRCTN-21047731.

Statistical analysis

Two by two tables were analysed by Fisher's exact test and non-parametric data by Mann-Whitney *U* test using the Statsdirect software package (version 2.6.5. www.statsdirect.com).

RESULTS

Demographic and biochemical profiles

As in the parent study, the two groups were well-matched in terms of age and gender distribution. Alcohol was the dominant etiologic agent and a majority in both groups were cigarette smokers (Table 1).

Antioxidant profiles at baseline and at 6 mo

Baseline levels of vitamin C, vitamin A, whole blood glutathione transferase and red cell glutathione transferase were similar between groups and were also within the reference range for population normal as reported by the Pancreatic laboratory of the Manchester Royal Infirmary (Table 2). Levels were towards the lower range of normal. Although median vitamin E, β -carotene and selenium levels were below the range for population normal in the placebo group, this difference was not significant compared to the antioxidant group at baseline.

Haemoglobin, white cell count and CRP were within normal levels in both groups.

At 6 mo (Table 3) there was significant elevation of vitamin C and selenium levels in the antioxidant group compared to baseline and also compared to placebo at 6 mo. Vitamin A and E levels were also significantly elevated in patients receiving antioxidant therapy compared to those receiving placebo at 6 mo. A similar pattern was seen for β -carotene although these values did not attain significance.

There was no difference in haemoglobin, white cell count or CRP at 6 mo between antioxidant therapy and placebo or between antioxidant therapy and baseline.

There were also no differences in opiate usage.

Cytokine profiles

There was no difference between the antioxidant group and placebo at baseline in any of the cytokines measured in this study (Table 4). Similarly, there was no difference between antioxidant and placebo at 6 mo and no difference in the antioxidant group at 6 mo compared to the same group at baseline. IL-1 β , IL-2, IL-4 and IL-10 me-

Table 1 Demographic profiles

Variables	Antioxidant (n = 7)	Placebo (n = 7)	P value
Age (yr), median (range)	46 (34-79)	46 (37-60)	0.92 (Mann-Whitney U)
Gender (male:female)	5:2	4:3	0.90 (Fisher's exact)
Aetiology	Alcohol 6; idiopathic 1	Alcohol 4; idiopathic 3	0.55 (Fisher's exact)
Disease duration (yr)	4 (1-5)	3 (2-13)	0.92 (Mann-Whitney U)
Body mass index (kg/m ²)	24.2 (18.8-36.7)	22.5 (22.9-32.8)	0.62 (Mann-Whitney U)
Alcohol (g/d), median (range)	175.5 (0-396)	138.6 (0-252)	0.43 (Mann-Whitney U)
Cigarette smoker (Y:N)	6:1	5:2	0.59 (Fisher's exact)
Diabetes mellitus (Y:N)	2:5	1:6	0.62 (Fisher's exact)
Faecal elastase (µg/g)	68 (15-500)	27 (15-500)	0.27 (Mann-Whitney U)
Opiate use (mg/d)	40 (30-300)	85 (0-120)	0.30 (Mann-Whitney U)

Laboratory reference range for faecal elastase report values < 200 µg/g as representing end-stage exocrine failure. All opiate intakes are reported as morphine equivalent.

Table 2 Baseline antioxidant profiles

Variables	Antioxidant (n = 7)	Placebo (n = 7)	Laboratory reference range	Median difference	P value (MWU)	95%CI
Vitamin C (µg/mL)	7.7 (0.7-13)	5.8 (2.4-9.9)	4-20	1.6	0.53	-3-6.1
Vitamin E (mg/L)	12.4 (5.4-20.9)	5.4 (3.6-15.2)	5.7-14.9	4.4	0.12	-2.6-11.1
β-carotene (mol/L)	35.9 (8-87)	11.6 (7-233)	19-254	15	0.55	-166-71
Vitamin A (mg/L)	0.60 (0.30-0.68)	0.40 (0.20-0.57)	0.4-1.2	0.16	0.07	-0.03-0.37
Selenium (µg/L)	82 (27-110)	49 (27-97)	83-152	27	0.22	-14-53
WGS (µmol/L)	1361 (1229-1682)	1336 (1149-1585)	1078-1753	62.5	0.73	-118-290
WGS/Hb (µmol/g)	9.2 (7.8-11.4)	9.7 (8.9-10.0)	7.5-12.2	-0.3	0.70	-1.4-1.5
WCC (10 ⁹ /L)	7.7 (6.4-10.3)	10 (5-15.9)	4-11	-1.4	0.38	-4.4-2.2
Hb (g/dL)	14.9 (13.4-15.8)	13.7 (12.4-16)	13-18	1	0.33	-0.6-2.1
CRP (mg/L)	3 (3-29)	7 (3-29)	0.3-5	-1	0.27	-7-3

WGS: Whole blood glutathione; WGS/Hb: Glutathione corrected for haemoglobin concentration; WCC: White cell count; Hb: Haemoglobin; CRP: C-reactive protein; MWU: Mann-Whitney U.

Table 3 Antioxidant profiles at 6 mo compared to baseline

Variables	Antioxidant (n = 7)	Placebo (n = 7)	P value (antioxidant vs baseline)	95%CI	P value (antioxidant vs placebo)	95%CI
Vitamin C (µg/mL)	17.6 (12.8-29.3)	4.8 (1.6-9.1)	0.001	-18.8-6.5	< 0.001	9.0-20.2
Vitamin E (mg/L)	17.8 (11.7-25.0)	5.0 (4.0-4.6)	0.160	-1.8-12.3	0.004	4.4-14.3
β-carotene (mol/L)	155.5 (23-478)	38.1 (8-204)	0.150	-189-35	0.244	-20-190
Vitamin A (mg/L)	0.5 (0.42-0.72)	0.3 (0.25-0.64)	0.910	-0.19-0.14	0.010	0.05-0.34
Selenium (µg/L)	109 (95-133)	48 (40-92)	0.007	-75-14	< 0.001	41-85
WCC (10 ⁹ /L)	6.7 (4.9-10.8)	7.4 (6-10.8)	0.330	-1.3-3.2	0.600	-3.3-1.9
Hb (g/dL)	14.2 (13.5-16.0)	12.7 (12.3-16.0)	0.510	-1-1.6	0.150	-1.3-2.6
CRP (mg/L)	3 (3-4)	6 (3-10)	0.190	0-3	0.070	-7-0
Opiate usage	20	55	0.210	-40-79	0.630	-56-60

WGS: Whole blood glutathione; WGS/Hb: Glutathione corrected for haemoglobin concentration; WCC: White cell count; Hb: Haemoglobin; CRP: C-reactive protein.

dian values were below the lower limit of the laboratory reference range at all sample points although individual patient sample values registered within the reference range. IL-6 and IL-8 values were within the reference range but towards the lower end at all sample points. VEGF showed higher values in the placebo group at both baseline and at 6 mo although this difference was not significant.

DISCUSSION

To the best of our knowledge, this study is the first to ex-

amine circulating cytokine levels in patients with chronic pancreatitis receiving antioxidant therapy and to compare these values to controls (also with CP) receiving matched placebo. When interpreting these findings, several important methodological sources of error should be emphasised. First, this is a small study with only 7 patients in each group. Thus it should be borne in mind that negative findings could represent a type II error. A second source of error is the possibility of technical compromise in assay methodology as samples were transferred for analysis. As the majority of readings were low, could deterioration in sample quality have affected the assays?

Table 4 Cytokine levels in patients receiving antioxidant therapy compared to placebo

Cytokine	Laboratory range	Antioxidant therapy baseline (pg/mL)	Placebo baseline (pg/mL)	<i>P</i> value	95%CI	Antioxidant therapy at 6 mo (pg/mL)	Placebo at 6 mo (pg/mL)	<i>P</i> value	95%CI
IL-1 β ¹	1.6-250	< 1.6	< 1.6			< 1.6	< 1.6		
IL-2	4.8-3000	2.6 (0-4.8)	3.1 (0-3.5)	0.83	-1.1-1.7	2.6 (0.0-4.8)	2.9 (0.0-3.2)	0.97	-2.6-2.3
IL-4	6.6-900	2.3 (2.2-6.6)	2.5 (2.1-6.6)	0.84	-3.7-3.9	2.5 (2.2-6.6)	2.8 (2.1-6.6)	0.81	-1.3-3.7
IL-6	1.2-900	1.9 (0.8-3.5)	1.8 (0.7-8.9)	0.99	-2.6-1.4	1.6 (1.0-2.3)	1.4 (0.9-7.6)	0.78	-3.9-0.7
IL-8	4.9-3000	10.1 (8.1-23.7)	8.5 (7.5-19.8)	0.46	-5.2-8.4	11.6 (8.1-19.3)	10.8 (6.6-18.6)	0.54	-2.7-18.7
IL-10 ²	1.8-1000	< 0.6	< 0.6			< 0.6	< 0.6		
TNF α	4.4-1500	3.5 (2.4-5.8)	3.5 (2.2-4.9)	0.71	-1.1-1.6	3.8 (2.4-4.7)	3.4 (2.5-4.5)	0.40	-0.7-1.1
IL-18	0-3000	388.6 (245.7-818.9)	457.9 (317.5-775.7)	0.71	-228-165	365.5 (211.5-879.8)	435.7 (349.1-570.1)	0.40	-238-299
VEGF	14.6-3000	116.2 (49.8-191.2)	243.1 (39.5-305.5)	0.32	-187-51	93.7 (35.1-173.9)	184.4 (34.1-299.9)	0.22	-206-34.7
EGF	2.9-900	28.4 (9.8-137.7)	23.4 (11.9-75.6)	0.99	-30.0-47.6	9.4 (3.1-65.2)	34.4 (2.3-139.8)	0.09	-66.8-5.5
MCP-1	13.2-1500	335.9 (267.1-423)	298.8 (229.5-685.1)	0.81	-134-106	296.5 (235.0-426.7)	326.4 (265.5-468.6)	0.38	-127-49

¹All patients had interleukin (IL)-1 β levels below the lower threshold of detection. IL-2: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.64 (95%CI: -1.5-2.5); IL-4: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.89 (95%CI: -3.7-3.7); IL-6: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.25 (95%CI: -0.4-1.7); IL-8: Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.69 (95%CI: -17.8-4.9); ²All patients had interleukin 10 below the lower threshold of detection. Transforming growth factor type β 1 (TGF β 1): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.46 (95%CI: -39-74); tumor necrosis factor α (TNF α): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.97 (95%CI: -1.0-1.4); vascular endothelial growth factor (VEGF): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.54 (95%CI: -43.8- 81.0); epidermal growth factor (EGF): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.05 (95%CI: -0.22-59.8); monocyte chemotactic protein-1 (MCP-1): Antioxidant group at 6 mo *vs* antioxidant group at baseline, *P* = 0.05 (95%CI: -50.1-100.9).

Whilst this possibility cannot definitively be excluded, the commercial laboratory which undertook these assays works closely with the clinical biochemistry department of the Manchester Royal Infirmary and regularly undertakes analysis of externally drawn samples. Sample extraction, storage and transfer were in full compliance with established protocols. Further, laboratory markers of the inflammatory response measured in-hospital such as the white cell count and CRP were also normal providing indirect support. A third caveat is that cytokine levels measured in blood may not necessarily reflect their activity at the pancreatic parenchymal level. For example, Noh and colleagues demonstrated that IL-8 concentrations are elevated (compared to non-disease controls) in pancreatic juice collected by duodenoscopy^[9].

Accepting these limitations, the present study does provide unique data on cytokine profiles in patients with chronic pancreatitis receiving antioxidant therapy and in a matched cohort receiving placebo and provides negative results which should be regarded as important pilot data. The first finding of interest is that at baseline, despite having radiological evidence of chronic pancreatitis, impairment of pancreatic exocrine function and a substantial requirement for opiate analgesia there was no elevation of circulatory pro- or anti-inflammatory cytokine levels. This is finding sits well with current paradigms of chronic pancreatitis which suggest that pain is not simply a product of inflammation and that it involves a complex interaction between inflammatory mediators and neural structures with alterations in nociception^[10,11]. For example, fractalkine is a cell surface membrane-spanning adhesion molecule that can be cleaved to produce a soluble neuro-modulatory chemokine which increases neuropathic pain through glial activation with expression correlating with the severity of pancreatic neuritis, fibrosis, intrapancreatic nerve fibre density and pain in chronic pancreatitis^[12]. Fractalkine may be a better disease-specific chemokine in

chronic pancreatitis although its relation to disease stage and response to therapy have yet to be elucidated^[13].

In relation to cytokine profiles in chronic pancreatitis reported in other studies, the levels of IL-18 in our study are similar to those reported by Schneider and colleagues^[14]. In terms of genotype, patients with alcohol-aetiology dominant, sporadic chronic pancreatitis do not have an increased frequency of functional polymorphisms in the *TGF β 1* gene, in the *IL-10* gene or in the intron 1 of the interferon-gamma gene^[15].

In this study there was no relation between antioxidant therapy and cytokine levels. Thus, the significant elevations in plasma levels of antioxidants seen in the treatment group (and also in the main ANTICIPATE study and in other studies of antioxidant therapy) do not appear to interact with circulating cytokines. The low levels of cytokines probably reflect the results of sampling of an out-patient based population with clinically quiescent disease and in particular without evidence of a systemic inflammatory response.

In conclusion, this study has measured antioxidant profiles in patients with chronic pancreatitis receiving antioxidant therapy and compared these to patients receiving matched placebo. Cytokine levels were low at baseline and at 6 mo despite a significant elevation in plasma antioxidants. The study also demonstrates that circulating cytokine levels are low suggesting that pain in this disease is not simply a manifestation of ongoing inflammation. It could be the result of the inflammation tissue damage caused long time ago.

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review of the final manuscript.

COMMENTS

Background

This study undertakes a subgroup analysis comparing pro- and anti-inflammatory cytokine levels in a sub-group of patients receiving either antioxidant therapy for chronic pancreatitis in the form of Antox (Pharmanord, Morpeth, United Kingdom) or matched placebo.

Research frontiers

The novel aspect of this study is that it is believed to be the first to examine pro- and anti-inflammatory cytokine levels in patients receiving antioxidant therapy for chronic pancreatitis and to compare these levels to those in patients receiving matched placebo.

Innovations and breakthroughs

The results show that pro-inflammatory cytokine levels were not elevated. This is potentially an important finding in that it shows that in patients with chronic pancreatitis, with established pain, inflammatory cytokine levels are not elevated.

Applications

The findings are preliminary and need to be reproduced in a larger validation dataset before more general acceptance.

Peer review

It is a very interesting paper. Considering that this paper employs patients from the ANTICIPATE study, it is desirable that the authors give the registration number of the main trial.

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