IL-32α-induced inflammation constitutes a link between obesity and colon cancer

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ABSTRACT
Growing evidence indicates that adipose tissue inflammation is an important mechanism whereby obesity promotes cancer risk and progression. Since IL-32 is an important inflammatory and remodeling factor in obesity and is also related to colon cancer (CC) development, the aim of this study was to explore whether IL-32 could function as an inflammatory factor in human obesity-associated CC promoting a microenvironment favorable for tumor growth. Samples obtained from 84 subjects (27 lean (LN) and 57 obese (OB)) were used in the study. Enrolled subjects were further subclassified according to the established diagnostic protocol for CC (49 without CC and 35 with CC). We show, for the first time, that obesity (p = 0.009) and CC (p = 0.026) increase circulating concentrations of IL-32α. Consistently, we further showed that gene (p < 0.05) and protein (p < 0.01) expression levels of IL-32α were upregulated in VAT from obese patients with CC. Additionally, we revealed that IL32 expression levels are enhanced by hypoxia and inflammation-related factors in HT-29 CC cells as well as that IL-32α is involved in the upregulation of inflammation (IL8, TNF, and CCL2) and extracellular matrix (ECM) remodeling (SPP1 and MMP9) genes in HT-29 cancer cells. Additionally, we also demonstrate that the adipocyte-conditioned medium obtained from obese patients stimulates (p < 0.05) the expression of IL32 in human CC cells. These findings provide evidence of the potential involvement of IL-32 in the development of obesity-associated CC as a pro-inflammatory and ECM remodeling cytokine.

Introduction
Although colorectal cancer (CRC) incidence has been declining over the past 20 y, it is still the second leading cause of cancer, prompting a significant morbidity and mortality. A connection between inflammation and CRC is well established, and in the last decade the role of distinct pro-inflammatory factors, cytokines, and other immune mediators have received a great deal of interest in the promotion and progression of CRC. In this regard, the characteristic chronic low-grade inflammatory state associated to obesity constitutes an important link between excess adiposity and CRC development. Epidemiological data evidence a significant association between increased body mass index (BMI) and several major cancers and specifically, more than 10% of cancers of the colon have been attributed to excess weight.

The molecular mechanisms by which obesity-associated inflammation promotes CRC development are still being uncovered but recent works have elucidated that the adipose tissue is a highly active endocrine organ that secretes a variety of adipokines with important roles in tumor initiation and progression. In this sense, our group has identified that the elevated levels of the recently described cytokine interleukin (IL)-32 in obesity promote adipose tissue inflammation and the remodeling of the extracellular matrix (ECM), suggesting its involvement in the development of obesity-associated comorbidities. IL-32 is produced by immune and non-immune cells including natural killer cells, monocytes, macrophages, T-lymphocytes, fibroblasts as well as epithelial and endothelial cells. Previous studies have demonstrated that different cytokines such as IL-1β, interferon-gamma (IFNγ) or tumor necrosis factor-alpha (TNF-α) induce the expression of IL-32 and, in turn, IL-32 also stimulates IL-8, IL-6, IL-1β, and TNF-α production in different cellular types via the activation of nuclear factor κB subunit 1 (NF-κB) and p38 mitogen-activated protein (MAP) kinase, constituting a classical pro-inflammatory mediator with relevant functions in angiogenesis, ECM remodeling and apoptosis.

In recent years, the role of IL-32 in certain forms of cancers has been reported with its expression being altered in different forms of gastric, lung, breast, colon or liver cancer. However, the results are contradictory with IL-32 exhibiting an oncogenic or a tumor suppressive role mainly due to the specific activities and properties of its nine isoforms generated by mRNA alternative splicing. Regarding CRC, IL-32 expression has been correlated with both lymph node and
organic metastasis development \(^{30}\) and a role in the regulation of the cancer stem cell-like properties promoting tumorigenesis of CRC in an autocrine and paracrine manner has been also described.\(^ {34}\) In this sense, the epithelial expression and circulating levels of IL-32\(\alpha\) were increased in patients with ulcerative colitis and Crohn’s disease.\(^ {25,35,36}\) In addition, IL-32 is a potent inducer of prostaglandin E2 release, an important factor for inflammation\(^ {23,37}\) and for the promotion of colon cancer (CC) cell growth.\(^ {38}\) On the contrary, an enhancement of TNF-\(\alpha\)-induced cell death in cases of CC as well as an inhibition of cancer cell growth by blocking the NF-\(\kappa\)B and the signal transducer and activator of transcription 3 (STAT3) pathways by IL-32\(\gamma\) has also been reported.\(^ {39,40}\)

Since IL-32 is reportedly an important inflammatory and remodeling factor in obesity\(^ {19}\) and is also related to CRC development, we hypothesized that the increased levels of IL-32 in obese patients may function as a link between obesity and CC development. Therefore, the aim of the present study was to investigate whether obesity can influence the circulating and expression levels of IL-32 in patients with CC, thereby promoting a microenvironment favorable for tumor growth. To gain insight into the molecular mechanisms involved, the effect of pro- and anti-inflammatory factors on the expression levels of IL-32 in cultures of human CC cells was further explored, and finally we also aimed to investigate whether IL-32 itself can regulate the inflammatory response in human CC cells.

## Results

### Obesity and colon cancer increase circulating concentrations and expression levels of IL-32\(\alpha\)

Baseline characteristics of the study sample are shown in Table 1. As expected, markers of adiposity were higher \((p < 0.001)\) in obese individuals compared with the lean volunteers. No differences in anthropometric measurements were detected between patients with or without CC. Obese patients with CC showed increased \((p < 0.001)\) levels of CRP compared with lean and obese patients without CC. Moreover, fibrinogen concentrations were higher \((p < 0.01)\) in obese patients with CC compared with obese patients without CC. No differences were found in the global WBC regarding obesity but patients with CC exhibited an elevated number of leucocytes \((p < 0.05)\) compared with those without CC. Significant differences in circulating IL-32\(\alpha\) concentrations between the experimental groups were observed, being significantly increased due to obesity \((p = 0.009)\) and CC \((p = 0.026)\) (Fig. 1A). No sexual dimorphism was found in plasma IL-32\(\alpha\) concentrations \((\text{male}: 129.9 \pm 15.9 \text{ arbitrary units (a.u.)}; \text{female}: 125.0 \pm 18.7 \text{ a.u.}; p = 0.964))\.

Since plasma levels of IL-32\(\alpha\) are increased by both obesity and CC, we further investigated gene and protein expression of IL-32\(\alpha\) in samples of VAT based on the relevance of this adipose tissue depot in obesity-associated inflammation and colon carcinogenesis. Obese patients with CC showed increased gene \((p < 0.05)\) and protein \((p < 0.01)\) expression levels of IL-32\(\alpha\) compared with obese patients without CC (Fig. 1B and C). To gain further insight into the presence of IL-32 in VAT, its localization was confirmed by immunohistochemistry (Fig. 1D). Both adipocytes and SVFC were immunopositive for IL-32\(\alpha\), although a strong staining in SVFC was observed and an enhanced expression was also detected in patients with CC.

In light of the significance of IL-32 in obese patients with CC and the potential role of insulin resistance in the development of CC, we also evaluated its gene expression levels in small intestine samples from obese patients, being significantly higher \((p < 0.01)\) in obese patients with type 2 diabetes compared with both impaired glucose tolerant and normoglycemic obese patients (Fig. 1E).

### Increased circulating levels of inflammation and ECM remodeling markers in obesity and CC are related to IL-32\(\alpha\)

Although the detailed mechanisms that mediate the obesity-driven effect on cancer development in humans are still poorly understood, chronic inflammation is known to participate in cancer progression. In this regard, significant differences in circulating IL-6 concentrations between the experimental groups were observed, being significantly increased due to obesity \((p = 0.002)\) and CC \((p < 0.001)\)

| Table 1. Anthropometric and biochemical characteristics of the subjects included in the study. |
|-----------------------------------------------|
| **Lean** | **Obese** | **p** | **p** | **p** |
| | | **Non-CC** | **CC** | **Non-CC** | **CC** | **Obesity** | **Cancer** | **Obesity \(\times\) Cancer** |
| **n (male, female)** | 13 (7, 6) | 14 (7, 7) | 36 (22, 14) | 21 (14, 7) | 0.518 | \(< 0.001\) | 0.925 |
| **Age (years)** | 53 ± 2 | 63 ± 3 | 55 ± 1 | 64 ± 3 | | | |
| **Body weight (kg)** | 62.7 ± 1.9 | 61.2 ± 5.1 | 83.2 ± 2.0 | 78.3 ± 2.2 | \(< 0.001\) | 0.577 | 0.320 |
| **Body mass index (kg/m\(^2\))** | 22.7 ± 0.9 | 22.4 ± 0.4 | 30.2 ± 0.7 | 29.6 ± 0.7 | \(< 0.001\) | 0.109 | 0.588 |
| **Estimated body fat (%)** | 29.9 ± 1.9 | 29.2 ± 1.5 | 37.4 ± 1.1 | 33.4 ± 1.4 | \(< 0.001\) | 0.155 | 0.370 |
| **Waist (cm)** | 83 ± 1 | 80 ± 1 | 99 ± 2 | 111 ± 2 | \(< 0.001\) | 0.241 | 0.180 |
| **Fasting glucose (mg/dL)** | 102 ± 4 | 141 ± 13 | 110 ± 5 | 128 ± 8 | 0.730 | \(< 0.001\) | 0.143 |
| **Free fatty acids (mg/dL)** | 12.7 ± 1.4 | 26.5 ± 2.4 | 15.4 ± 1.2 | 22.2 ± 1.7 | 0.570 | \(< 0.001\) | 0.064 |
| **Triglycerides (mg/dL)** | 87 ± 10 | 112 ± 11 | 117 ± 9 | 121 ± 20 | 0.747 | 0.752 | 0.685 |
| **C-reactive protein (mg/L)** | 0.20 ± 0.13 | 1.10 ± 0.96 | 1.17 ± 0.08 | 8.48 ± 1.84\(^a\) | 0.008 | \(< 0.001\) | 0.031 |
| **Fibrinogen (mg/dL)** | 337 ± 27 | 277 ± 26 | 300 ± 17 | 451 ± 30\(^b\) | 0.159 | 0.418 | 0.033 |
| **Carcinomembroncy antigen (ng/mL)** | 1.58 ± 0.32 | 2.55 ± 0.44 | 1.68 ± 0.28 | 8.41 ± 2.60 | 0.267 | 0.021 | 0.401 |
| **Leucocyte \((\times 10^3)/\text{L})** | 6.17 ± 0.91 | 8.19 ± 1.14 | 6.22 ± 0.31 | 7.73 ± 0.83 | 0.823 | 0.024 | 0.653 |

Data are mean ± SEM. CC, colon cancer. Statistical differences were analyzed by two-way ANCOVA and one-way ANCOVA followed by Tukey’s post-hoc tests as appropriate.

\(^a\) \(p < 0.001\) vs lean non-CC, lean-CC and obese non-CC.

\(^b\) \(p < 0.01\) vs obese non-CC.
In this sense, obese patients with CC exhibited higher (\( p < 0.026 \)) plasma levels of IL-8 compared with normoponderal patients with CC (Fig. 2B). Circulating levels of the anti-inflammatory cytokine IL-4 were also affected by obesity (\( p < 0.038 \)) and CC (\( p < 0.046 \)) but in an opposite way, exhibiting decreased circulating levels in obese and CC patients (Fig. 2C). IL-13 concentrations were diminished (\( p = 0.016 \)) in obese patients, but no differences were detected regarding the presence or not of CC (Fig. 2D).

Importantly, circulating levels of IL-32 were significantly associated with the concentrations of the pro-inflammatory cytokine IL-6 (\( r = 0.37; \ p = 0.046 \)).

Moreover, changes in the ECM composition of adipose tissue in obesity greatly influence the metabolism and growth of tumor cells, providing a favorable area for tumorigenesis. In this regard, we found that obesity significantly increased (\( p < 0.05 \)) plasma levels of VEGFA and WISP1 and that patients with CC showed decreased (\( p = 0.021 \)) circulating concentrations of SPARC (Fig. 3). OPN concentrations were significantly increased due to both obesity (\( p < 0.01 \)) and CC (\( p < 0.05 \)). We also found a positive correlation of IL-32\( \alpha \) concentrations with serum levels of WISP1 (\( r = 0.58; \ p = 0.007 \)) and OPN (\( r = 0.40; \ p = 0.043 \)) as well as a negative association with SPARC concentrations (\( r = -0.60; \ p = 0.008 \)).

**Inflammatory-related factors and hypoxia increase IL32 gene expression levels in HT-29 cells**

We next evaluated if well-known inflammatory factors that are increased in obesity influence IL32 expression in HT-29 cells.
TNF-α treatment dramatically increased \((p < 0.001)\) the mRNA levels of IL32 and, in the same line, gene expression levels of IL32 were induced by LPS \((p < 0.01)\). No significant changes were observed on IL32 expression in HT-29 cells after their stimulation with the anti-inflammatory cytokines IL-4 and IL-13 (Fig. 4C and D). Next, we showed that gene expression levels of IL32 exhibited a strong upregulation \((p < 0.001)\) in HT-29 cells under hypoxia mimicked by the incubation with the divalent transition-metal ion cobalt at 100 mM (Fig. 4E).

**IL-32α induces the expression of inflammation and ECM remodeling markers in HT-29 cells**

We further explored whether IL-32α itself can activate the expression of genes involved in the inflammatory response and ECM remodeling in HT-29 cells. Cells were stimulated with increasing concentrations of IL-32α for 24 h. As shown in Fig. 5, IL-32α treatment significantly enhanced the mRNA levels of the inflammatory factors TNF \((p < 0.01)\), CCL2 \((p < 0.05)\) and IL8 \((p < 0.05)\). We also detected a strong upregulation \((p < 0.001)\) of the ECM remodeling genes SPP1 and MMP9 after IL-32α treatment (Fig. 5). Unexpectedly, no differences were found in the regulation of IL1B and VEGFA after treatment with IL-32α.

We also explored the impact of IL-32α on the modulation of the expression of specific key genes controlling cell proliferation, growth and adhesion in CC. In this sense, IL-32α treatment significantly reduced gene expression levels of the tumor suppressor gene APC \((p < 0.05)\) and increased mRNA levels of CTNNB1 \((p < 0.05)\). However, no significant differences in the expression levels of KLF4, COX2, HIF1A, TGFβ, and MUC2 were found after treatment with IL-32α (Fig. S1).

**ACM upregulates gene expression levels of IL32 in HT-29 cells**

To explore molecular mechanisms behind the adipocyte-tumoral cell crosstalk, we analyzed the effect of ACM in the expression of IL32 in HT-29 cells. Interestingly, a significant increase \((p < 0.05)\) in the expression levels of IL32 in HT-29 cells preincubated with the adipocyte-derived factors obtained from obese volunteers compared with the tumoral cells treated with control media was observed (Fig. 6).

**Discussion**

Growing evidence indicates that inflammation is a central mechanism through which obesity promotes cancer risk and progression.\textsuperscript{12,41} In addition to the tumor-promoting effects of systemically dysregulated adipokines as well as metabolic and inflammatory mediators, obesity also promotes tumor progression at the local level via adipose tissue inflammation and associated alterations in the microenvironment.\textsuperscript{15,41} In this context,
increased levels of the pro-inflammatory cytokine IL-32 have been found in obesity\(^1\) as well as in several major cancers.\(^{27,29,31}\) The present study provides evidence that the increased IL-32 expression in obesity is related to CC development. In this regard, we found, for the first time, that both obesity and CC increased circulating levels of IL-32. Consistently, we further showed that the \(\text{IL32}^\) gene and protein expression levels in VAT were also upregulated in obese patients with CC. Additionally, we revealed that \(\text{IL32}^\) expression levels are regulated by hypoxia and inflammation-related factors in human CC cells as well as that IL-32 is involved in enhancing inflammation and ECM remodeling. Subsequently, we also demonstrate that ACM stimulates the expression of \(\text{IL32}^\) in human CC cells.

IL-32 is a multifaceted cytokine with relevant cell functions; inducer of proinflammatory cytokines,\(^43\) apoptosis,\(^42\) and differentiation.\(^43\) In the present study, we have found that circulating concentrations of IL-32\(^\alpha\) are increased due to both obesity and CC, suggesting a link between obesity and this relevant comorbidity. Increased circulating levels of IL-32 have been described in different tumoral types\(^{27-29}\) although results are contradictory because of the different activity of the major isoforms of IL-32. In this sense, IL32-\(\beta\) and IL-32\(^\gamma\) have been related to caspase-8- and caspase-3-induced apoptosis whereas this effect was not observed for the IL-32\(^\alpha\) isoform.\(^{21,32,44}\) Importantly, IL-32\(^\beta\)-induced cell death can be rescued through an enhanced expression of IL-8, a cytokine involved in cell survival whereas IL-32\(^\gamma\), the most potent isoform, has the capacity to block the IL-8-survival signaling pathway.\(^32\) An anticancer activity of IL-32\(^\gamma\) has been described to induce CC cell death \textit{in vitro} and tumor regression \textit{in vivo} by enhancing TNF-\(\alpha\) expression\(^40\) and by decreasing the activity of the STAT3 pathway, highly involved in the inflammation-associated carcinogenesis in CC.\(^39\) However, an induction of metastasis by the overexpression of IL-32 in CRC has been observed\(^30\) and a marked expression of IL-32\(^\alpha\) has been found in colon mucosa from patients with different inflammatory bowel diseases such as ulcerative colitis and Crohn’s disease.\(^36\) This paradoxical effect has been explained in transgenic mice expressing human IL-32\(^\gamma\), in which the exacerbated acute inflammation found in early stages of the development of ulcerative colitis is reduced after later stages of the disease.\(^35\) IL-32\(^\gamma\) is able to increase IL-10 expression, which is involved in the decrease of TNF-\(\alpha\), IL-6 and IL-32 itself, causing a decrease in the inflammatory state.\(^35\) Therefore, IL-32\(^\gamma\) may be viewed as a cytokine that worsens as well as protects the intestinal integrity.

High circulating levels and expression of IL-32 have been associated with a poor prognosis of gastric cancer mainly as a result of an induction of inflammatory and remodeling factors.\(^{27,45}\) In this context, obese patients with CC included in our study also showed increased levels of the relevant inflammatory markers IL-6, IL-8, and OPN and their strong association with IL-32 levels underscores the involvement of this cytokine in obesity- and CC-associated inflammation. Obese

\[\text{Figure 3. Effect of obesity and colon cancer (CC) on extracellular matrix remodeling factors. Circulating concentrations of (A) vascular endothelial growth factor A (VEGFA), (B) WNT1 inducible signaling pathway protein 1 (WISP1), (C) secreted protein acidic and cysteine rich (SPARC) and (D) secreted phosphoprotein 1 [osteopontin (OPN)] of lean (LN) and obese (OB) volunteers classified according to the presence or not of CC. Bars represent the mean ± SEM. Differences between groups were analyzed by two-way ANCOVA.}\]
patients showed increased levels of WISP1, a novel adipokine linking obesity to inflammation and insulin resistance.46 The association between IL-32 and WISP1 found in our study strengthens the involvement of IL-32 in obesity-associated inflammation. We also showed reduced levels of SPARC in patients with CC as well as a negative association between SPARC and IL-32. Although the role of SPARC in cancer remains controversial and it is highly cancer-type dependent,
SPARC has been considered to be a tumor suppressor in CC, suggesting a role for IL-32 in colon carcinogenesis. Adipose tissue represents an extremely active endocrine organ. The expanded and dysfunctional adipose tissue in obesity plays an important role for tumor development due to its ability to secrete inflammatory cytokines. In our study, obese patients with CC exhibited increased gene and protein expression levels of IL-32 in VAT compared with obese patients without CC, suggesting that VAT in obesity might be involved in establishing a microenvironment favorable for promoting CC growth and progression through the expression of IL-32. In this regard, diverse dysregulated adipokines and inflammatory factors synthesized and secreted by the adipose tissue in obesity have been linked to CC development and progression probably by inducing inflammation and modifying cancer cell behavior.

A potential limitation of the study is the classification of the volunteers according to BMI, which may misclassify obesity in some subjects with a proportion of obese individuals by body fat actually not being identified. Body composition assessment was not available for all the subjects included in the study. Therefore, to reduce the potential error, we estimated adiposity by using CUN-BAE, a validated tool designed by our group precisely to be applied for estimating body adiposity in adults in these circumstances with low error rate and good accuracy.

Inflammatory pathways have emerged as promising targets for cancer therapy. Therefore, new biomarkers of adipose tissue inflammation to identify obese patients at increased risk for cancer development could represent novel therapeutic targets. In the present study, the upregulated levels of IL-32 in patients with obesity and CC as well as its capacity to induce pro-inflammation and ECM remodeling genes suggest the involvement of IL-32 in the development of obesity-associated CC. Further studies in larger cohorts to better understand the implication of IL-32 in obesity-associated CC are warranted. Furthermore, the thorough characterization of IL-32 isoforms and the possibility to modulate their expression in a tissue-specific way may also represent a novel strategy in cancer therapies.

**Material and methods**

**Patient selection**

Blood samples from 84 subjects [27 lean (LN) and 57 obese (OB)] recruited from healthy volunteers and patients attending the Departments of Endocrinology & Nutrition and Surgery at the Clínica Universidad de Navarra were used in the study. Volunteers underwent a clinical assessment including medical history, physical examination and co-morbidity evaluation by a multidisciplinary consultation team. Patients were classified as LN or OB according to BMI (LN: BMI < 25 kg/m² and OB: BMI ≥ 30 kg/m²). BMI was calculated as weight in kilograms divided by the square of height in meters and body fat percentage (BF) was estimated using the Clínica Universidad de Navarra.
Navarra-Body Adiposity Estimator (CUN-BAE). Waist circumference was measured at the midpoint between the iliac crest and the rib cage on the midaxillary line. Enrolled subjects were further subclassified according to the established diagnostic protocol for CC [49 without CC (non-CC) and 35 with CC]. Clinicopathological characteristics of the subjects with CC included in the study are shown in Table S1.

Adipose tissue samples were collected from patients undergoing Nissen fundoplication (for LN volunteers), Roux-en-Y gastric bypass [RYGB (for morbid obesity treatment in OB subjects)] and curative resection for primary colon carcinoma (for CC treatment) at the Clínica Universidad de Navarra. In addition, an intraoperative biopsy from the small intestine was performed in the obese patients during RYGB to analyze IL32 gene expression levels. The control volunteers were healthy, were not on medication, and had no signs or clinical symptoms of cancer, liver alteration or type 2 diabetes. The experimental design was approved, from an ethical and scientific standpoint, by the Hospital’s Ethical Committee responsible for research and the written informed consent of participants was obtained.

**Analytical procedures**

Plasma samples were obtained by venipuncture after an overnight fast to avoid potential confounding influences due to hormonal rhythmicity. Glucose was analyzed by an automated analyzer (Hitachi Modular P800, Roche, Basel, Switzerland). Serum concentrations of triglycerides and free fatty acids (FFA) were measured by enzymatic methods using commercially available kits (InfinityTM, Thermo Electron Corporation, Melbourne, Australia). The carcinoembryonic antigen (CEA), high sensitivity C-reactive protein (CRP) and fibrinogen concentrations were determined as previously reported. White blood cell (WBC) count was measured using an automated cell counter (Beckman Coulter, Inc., Fullerton, CA). Commercially available ELISA kits were used to assess circulating levels of IL-4, IL-6, IL-8, IL-13, secreted protein acidic and cysteine rich (SPARC), secreted phosphoprotein 1 [osteopontin (OPN)], vascular endothelial growth factor A (VEGFA) and WNT1 inducible signaling pathway protein 1 (WISP1) (RayBiotech, Inc., Norcross, GA), according to the manufacturer’s instructions. The intra- and inter-assay coefficients of variation were <10 and <12% for all analyzed molecules.

**Real-time PCR**

The transcript levels for adenomatosis polyposis coli tumor suppressor (APC), β-catenin (CTNNB1), monocyte chemoattractant protein-1 (CCL2), cycloxygenase-2 (COX2), hypoxia-inducible factor 1α (HIF1A), IL1B, IL8, IL32, krüppel-like factor 4 (KLF4), matrix metalloproteinase 9 (MMP9), mucin 2 (MUC2), secreted phosphoprotein 1 (SPP1), transforming growth factor β (TGFβ), TNF, and VEGFA were quantified by Real-Time PCR (7300 Real Time PCR System, Applied Biosystems, Foster City, CA) as described previously. Primers and probes (Table S2) were designed using the software Primer Express 2.0 (Applied Biosystems) and purchased from Genosys (Sigma-Aldrich, Madrid, Spain). Primers or TaqMan® probes encompassing fragments of the areas from the extremes of two exons were designed to ensure the detection of the corresponding transcript avoiding genomic DNA amplification.

**Western blot studies**

Visceral (VAT) adipose tissue were homogenized and protein content was measured as described previously. Adipose tissue (30 µg) or diluted (1:100) blood samples were run out in Any kD™ Mini-Protein® TGX™ precast polyacrylamide gels (Bio-Rad Laboratories, Inc., Hercules, CA), subsequently transferred to nitrocellulose membranes and blocked in Tris-buffered saline (TBS) (50 mmol/L Tris, 0.5 mol/L NaCl, pH 7.36) with 0.05% Tween 20 containing 5% non-fat dry milk for 1 h at room temperature (RT).

Blots were incubated with a rat monoclonal anti-human IL-32α antibody (R&D Systems, Minneapolis, MN) diluted 1:10,000. The antigen–antibody complexes were visualized using horseradish peroxidase-conjugated anti-mouse IgG antibodies (1:20,000) and the enhanced chemiluminescence Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, Rockford, IL). The intensity of the bands was determined by densitometric analysis with the ChemiDoc™ MP imagining system and the Image Lab 4.0.1 software (Bio-Rad) and normalized with total protein values. All assays were performed in duplicate.

**Histological analysis of IL-32α**

Sections (6 µm) of formalin-fixed paraffin-embedded VAT were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol and treated with 3% H2O2 (Sigma) in absolute methanol for 10 min at RT to quench endogenous peroxidase activity. Then, slides were blocked during 1 h with 1% BSA (Sigma) diluted in TBS to prevent non-specific adsorption. Sections were incubated overnight at 4°C with a rat monoclonal anti-human IL-32α antibody (R&D Systems, Minneapolis, MN) diluted 1:50 in TBS. After washing with TBS, slides were incubated with Dako Real™ EnVision™ HRP-conjugated anti-mouse (Dako, Glostrup, Denmark) for 1 h at RT. After washing in TBS, the peroxidase reaction was visualized with a 3,3’-diaminobenzidine (DAB, Amersham Biosciences, Buckinghamshire, UK)/H2O2 solution (0.5 mg/mL DAB, 0.03% H2O2 diluted in 50 mmol/L Tris–HCl, pH 7.36), as chromogen and Harris hematoxylin solution (Sigma) as counterstaining. Finally, sections were dehydrated, mounted using DePeX mounting medium (Serva, Heidelberg, Germany) and observed under a Zeiss Axiowert CFL optic microscope (Zeiss, Göttingen, Germany). Negative control slides without primary antibody were included to assess non-specific staining.

**Adipocyte and HT-29 cell cultures**

Human stromovascular fraction cells (SVFCs) were isolated from omental adipose tissue from obese normoglycemic subjects as described previously. SVFC were seeded at 2 × 10⁵ cell/well and grown in adipocyte medium [DMEM/F-12 [1:1] (Invitrogen), 17.5 µL/L glucose, 16 µmol/L L-biotin, 18 µmol/L panthotenate, 100 µmol/L ascorbate, and antibiotic–antimycotic] supplemented with 10% newborn calf serum (NCS).
After 4 d, the medium was changed to adipocyte medium supplemented with 3% NCS, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 0.1 μmol/L dexamethasone, 1 μmol/L BRL49653 and 10 μg/mL insulin. After a 3-d induction period, cells were fed every 2 d with the same medium but without IBMX and BRL49653 supplementation for the remaining 7 d of adipocyte differentiation. Adipocyte-conditioned media (ACM) was prepared by collecting the supernatant from differentiated adipocytes. The ACM was then centrifuged, diluted (20% and 40%) and frozen at −80°C.

The intestinal epithelial cell line HT-29, derived from a human colorectal adenocarcinoma, was obtained from ATCC® (HTB-38™, Middlesex, UK) and cultured according to the manufacturer’s instructions. Briefly, cells were seeded at 3 × 10⁵ cells/well and grown in McCoy’s 5A medium with L-glutamine (Sigma) supplemented with 10% fetal bovine serum and antibiotic-antimicotic at 37°C for 24 h. HT-29 cells were serum-starved for 24 h and then treated with increasing concentrations of LPS (10, 100, and 1,000 ng/mL) (Sigma), TNF-α (1, 10, and 100 ng/mL) (Sigma), IL-4 (10, 100, and 1,000 ng/mL) (R&D Systems), IL-10 (10, 100, and 1,000 ng/mL) (R&D Systems), IL-32α (R&D Systems) (10, 100, and 1,000 ng/mL), CoCl₂ (100 and 200 nmol/L) or ACM (20% and 40%) for 24 h.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Due to their non-normal distribution CRP concentrations were logarithmically transformed. The normal distribution of the other variables was adequate for the use of parametric tests. Differences between groups were assessed by two-way ANOVA and one-way ANOVA followed by Tukey’s or Dunnet’s post-hoc tests as appropriate. Differences between groups adjusted for age were analyzed by analysis of covariance (ANCOVA). Associations between two variables were computed by Pearson (r) correlation coefficient. Multiple linear regression analysis was performed to evaluate the independent relationship of the studied variables. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS, Chicago, IL). A p value < 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

V.C. designed the study, collected, and analyzed data, wrote the first draft of the manuscript, contributed to discussion, and reviewed the manuscript. J.G.-A., A.R., and S.B. collected and analyzed data, contributed to discussion, and reviewed the manuscript. J.L.H.-L., J.B., F.R., V.V., and J.S. enrolled patients, collected data, contributed to discussion, and reviewed the manuscript. B.R., V.A.O., R.M., and C.S. collected data, contributed to discussion, and reviewed the manuscript. G.F. designed the study, collected patients, collected and analyzed data, wrote the first draft of the manuscript, contributed to discussion, and reviewed the manuscript. V.C. and G.F. are guarantors for the contents of the article and had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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