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Quantification and Potential Functions of Endogenous Agonists of Transient Receptor Potential Channels in Patients With Irritable Bowel Syndrome

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Quantification and Potential Functions of Endogenous Agonists of Transient Receptor Potential Channels in Patients With Irritable Bowel Syndrome

Short title: endogenous agonists of transient receptor channels in IBS

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Abbreviations: acetonitrile (ACN), 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), arachidonic acid (AA), Colorectal distension (CRD), cyclooxygenase (COX), cytochrome epoxygenase (CYPe), dihomo γ -linolenic acid (DGLA), docosahexaenoic acid (DHA), dorsal root ganglia (DRG), eicosapentaenoic acid (EPA), epoxyeicosatrienoic acid (-EET), Fast Blue (FB), formic acid (FA), Hank's Balanced Salt Solution (HBSS), healthy control (HC), 17-hydroxy-docosahexaenoic acid (17-HDoHE), 18-hydroxyeicosapentaenoic acid (18-HEPE), hydroxyeicosatetraenoic acid (HETE), hydroperoxyeicosatetraenoic acid (HpETE), irritable

Bowel Syndrome (IBS), diarrhea-predominant IBS (IBS-D), constipation-predominant IBS (IBS-C), mixed bowel habit IBS (IBS-M), lipoxin A4 deuterated (LxA₄-d₅), leukotriene B₄ (LTB₄), lipoxygenase (LOX), liquid chromatography/tandem mass spectrometry (LC-MS/MS), Methanol (MeOH), Polyunsaturated fatty acid (PUFA), Prostaglandin A₁ (PGA₁), Proteinase-Activated Receptor-2 (PAR₂), PAR₂-agonist peptide (PAR₂-AP), PAR₂-inverse peptide (PAR₂-IP), resolvin (Rv), tetrodotoxin-resistant (TTX-R), Transient receptor potential (TRP), Transient Receptor Potential Ankyrin-1 (TRPA1), transient receptor potential vanilloid (TRPV), visceral motor response (VMR).

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Pauline Le Faouder: acquisition of data; analysis of data; drafting of the manuscript

Nicholas A. Veldhuis: acquisition of data; analysis of data

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Background & Aims: In mice, activation of the transient receptor potential cation channels (TRP) TRPV1, TRPV4, and TRPA1, causes visceral hypersensitivity. These receptors and their agonists might be involved in development of irritable bowel syndrome (IBS). We investigated whether polyunsaturated fatty acid (PUFA) metabolites, which activate TRPs, are present in colon tissues from patients with IBS and act as endogenous agonists to induce hypersensitivity.

Methods: We analyzed colon biopsy samples from 40 patients with IBS (IBS biopsies) and 11 healthy individuals undergoing colorectal cancer screening (controls), collected during colonoscopy at the University of Bologna, Italy. Levels of the PUFA metabolites that activate TRPV1 (12-HpETE, 15-HETE, 5-HETE, LtB4), TRPV4 (5,6-EET; 8,9-EET), and TRPA1 (PGA1, 8-iso-PGA2, 15-d-PGJ2) were measured in biopsies and their supernatants using liquid chromatography and tandem mass spectrometry; we also measured levels of the PUFA metabolites PGE₂ and resolvins. C57Bl6 mice were given intrathecal injections of small interfering (si)RNAs to reduce levels of TRPV4, or control siRNAs, along with colonic injections of biopsy supernatants; visceral hypersensitivity was measured based on response to colorectal distension. Mouse sensory neurons were cultured and incubated with biopsy supernatants and lipids extracted from biopsies or colons of mice. Immunohistochemistry was used to detect TRPV4 in human dorsal root ganglia samples (from the National Disease Research Interchange).

Results: Levels of the TRPV4 agonist 5,6-EET, but not levels of TRPV1 or TRPA1 agonists, were increased in IBS biopsies compared with controls; increases correlated with pain and bloating scores. Supernatants from IBS biopsies, but not from controls, induced visceral hypersensitivity in mice. siRNA knockdown of TRPV4 in mouse primary afferent neurons

inhibited the hypersensitivity caused by supernatants from IBS biopsies. Levels of 5,6-EET and 15-HETE were increased in colons of mice with, but not without, visceral hypersensitivity. PUFA metabolites extracted from IBS biopsies or colons of mice with visceral hypersensitivity activated mouse sensory neurons in vitro, by activating TRPV4. Mouse sensory neurons exposed to supernatants from IBS biopsies produced 5,6-EET via a mechanism that involved the proteinase activated receptor-2 and cytochrome epoxygenase. In human dorsal root ganglia, TPV4 was expressed by 35% of neurons.

Conclusion: Colon tissues from patients with IBS have increased levels of specific PUFA metabolites. These stimulate sensory neurons from mice, and generate visceral hypersensitivity via activation of TRPV4.

KEYWORDS: pain, proteolytic activity, protease, PAR2

Introduction

Surveys of Western populations have revealed IBS in 15–20% of adolescents and adults, with a higher prevalence in women; the prevalence is variable in other populations¹. Considered as a functional bowel disorder, IBS is characterized by abdominal discomfort or pain, associated with constipation, diarrhea, or mixed bowel habit (IBS-C, IBS-D and IBS-M, respectively)¹. Recently, low-grade inflammation has been described to be associated with IBS¹. Among the inflammatory mediators studied in IBS, an increase in protease concentration or in proteolytic activity appears as a common feature to all IBS sub-groups^{2, 3}. Proteolytic activity released from IBS patient tissues is able to activate sensory neurons, and generates visceral hypersensitivity in mice, through a mechanism involving the activation of Proteinase-Activated Receptor-2 (PAR₂)³. Further studies have demonstrated in a mouse model, that PAR₂-induced visceral hypersensitivity is dependent on the activation of TRPV4 (transient receptor potential vanilloid-4)⁴. TRPV4 activation is not only a downstream effector of PAR₂ signaling, but is also responsible for serotonin- or histamine-induced visceral hypersensitivity⁵. These results place TRPV4, a cation channel widely expressed in the gastrointestinal tract⁶, at the crossroads of signaling events associated with visceral hypersensitivity. Likewise, other members of the “Transient Receptor Potential” (TRP) family: TRPV1 and TRPA1 (Transient Receptor Potential Ankyrin-1) seem to be implicated in visceral hypersensitivity in mouse models^{7, 8}. Whether TRP channels are playing such role in IBS has not been addressed yet, and the presence and functionality of endogenous agonists of these three TRPs in IBS have never been reported.

A common class of molecules: polyunsaturated fatty acid (PUFA) metabolites can signal through TRPV1, TRPV4 and TRPA1⁹ (Supplementary Figure 1). *In vitro* studies have shown that cytochrome epoxygenase (CYPe) products of arachidonic acid (AA) metabolism, 5', 6'-epoxyeicosatrienoic acid (5,6-EET) and 8, 9-EET, activate TRPV4^{10, 11}. Metabolites of

AA such as 12-hydroperoxyeicosatetraenoic acid (12-HpETE), 5-hydroxyeicosatetraenoic acid (HETE), 15-HETE and leukotriene B₄ (LTB₄) produced by lipoxygenases (LOX) have been characterized *in vitro* as potential TRPV1 agonists¹². Prostaglandin A₁ (PGA₁), a cyclooxygenase (COX) product of dihomo- γ -linolenic acid (DGLA) is able to activate TRPA1¹³. Similarly, cyclopentenone prostaglandins, such as 8-iso prostaglandin A₂ (8-iso-PGA₂) and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), can signal to cells through TRPA1 activation^{13, 14}. Resolvins (Rv), another class of PUFA metabolites formed by the metabolism of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by LOX enzymes, have been described as TRPs inhibitors. RvD1 can inhibit TRPV4 and TRPA1 and RvD2 inhibits TRPA1 and TRPV1¹⁵.

We have focused our attention on PUFA metabolites and TRP channels in the context of IBS. Here, we provide evidences that link PUFA metabolites, TRPV4 activation and IBS-D. Our results suggest that PUFA metabolites produced by colonic tissues of IBS-D patients activate TRPV4 to induce hypersensitivity symptoms.

Methods

Animals

Animals (supplementary method 2) have been used for sensory neurons primary culture (supplementary method 3) to perform lipid quantification (supplementary method 4 and 5) and calcium flux experiment (supplementary method 6) and colorectal distention experiment (supplementary method 7).

Patients

Patients (see supplementary method 8) underwent colonoscopy and in all cases, we obtained six mucosal biopsies from the proximal descending colon. One biopsy was sent to the Pathology Department for exclusion of microscopic colitis or other microscopic tissue

abnormalities. One biopsy was snap-frozen in liquid nitrogen for lipid extraction and PUFA quantification (Table 1, supplementary method 4 and 5). Four biopsies were used to obtain mucosal mediator release (Table 1)². Supernatant were used to quantify PUFA metabolites (supplementary method 4 and 5) and to test their effect in mouse visceral sensitivity (supplementary method 7) and PUFA metabolites released by mouse sensory neurons (supplementary method 3).

Results

Increased TRPV4 agonist in biopsies from IBS-D patients

Potential TRP channel agonists and inhibitors were quantified in biopsies of IBS patients and HC using liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Figure 1A-E). In diarrhea-predominant IBS (IBS-D) patients the quantity of 5,6-EET, an eicosanoid described as a TRPV4 agonist, was significantly increased compared to HC (Figure 1A). No significant difference was observed between constipation-predominant IBS (IBS-C) or mixed bowel habit IBS (IBS-M) and HC (Figure 1A). The concentration of 5,6-EET in biopsies of IBS patients and HC significantly correlated with the abdominal pain severity and frequency score and with the bloating severity and frequency score (Figure 2A). Concerning the quantification of 8, 9-EET, an eicosanoid also described as a TRPV4 agonist, no significant difference was observed between groups of IBS patients and HC (Figure 1A). No significant difference in the quantity of potential TRPV1 agonists (LTB₄, 5-HETE, 12-HETE and 15-HETE) was detected between IBS patients and HC (Figure 1B). Prostaglandin A₁, a TRPA1 agonist, was significantly decreased in IBS-C and IBS-D patients, compared to HC (Figure 1C). The quantity of 15d-PGJ₂ and 8-isoPGA₂, two eicosanoids identified as TRPA1 agonists, was unchanged between the different groups of patients and HC (Figure 1C). RvD1 and RvD2 were not detected in biopsies. Precursors of RvD (17-HDoHE) and of RvE (18-HEPE) were

detected in biopsies, but no significant difference between groups of IBS patients and HC was quantified (Figure 1D). PGE₂, a n-6 PUFA metabolite produced by the metabolism of AA by COX, was increased only in biopsies of IBS-D patients compared to HC (Figure 1E). The concentration of PGE₂ significantly correlated with the abdominal pain frequency score but not with the abdominal severity score or the bloating score (Figure 2B).

In supernatants from IBS-D patient biopsies or from HC biopsies, the quantity of TRPV4 agonists was not detectable (Supplementary Figure 2A). Quantities of TRPV1 and TRPA1 agonists or precursors of Rv were identical in supernatants from HC and IBS-D biopsies (Supplementary Figure 2B-D). PGE₂ was increased in supernatants of biopsies from IBS-D patients compared to HC (Supplementary Figure 2E).

Supernatants from IBS patient biopsies induced visceral hypersensitivity in mice and increased concentrations of TRP agonists

Intracolonic administration of supernatants from IBS-D patients induced hypersensitivity in response to colorectal distension, characterized by an increase in the intensity of visceral motor response (VMR) (Figure 3A). In contrast, mice that had received intracolonic administration of supernatants from control patients demonstrated levels of VMR similar to the baseline (Figure 3A). Potential TRP channel agonists were quantified in colons of these hypersensitive mice (administered with supernatants of biopsies from IBS-D patients) and in colons of normo-sensitive mice (administered with supernatants of biopsies from HC), using LC-MS/MS (Figure 3B-F). As observed in human tissues, the TRPV4 agonist 5,6-EET was significantly increased in the colon of hypersensitive mice compared to colons from normo-sensitive mice (Figure 3B). In hypersensitive mice, the concentration of TRPV1 agonist 15-HETE in colonic tissues was significantly increased compared to normo-sensitive mouse colons (Figure 3C). As in human, PGE₂ was increased in colons of hypersensitive mice,

compared to normo-sensitive mice (Figure 3F). None of the other PUFA metabolites concentrations were significantly different in the colon of hypersensitive mice, compared to normo-sensitive mice (Figure 3 B-E).

Lipids extracted from biopsies of IBS-D patients or hypersensitive mice activated sensory neurons in a TRPV4-dependent manner

To determine if 5,6-EET and PUFA metabolites can signal to sensory nerves innervating the colon, we examined the effects of pure 5,6-EET and the effects of the whole lipids extracted from biopsies of control and IBS patients or from the colon of mice that have normal visceral nociception or that are hypersensitive. In wild type mice, 5,6-EET stimulated a rapid increase in $[Ca^{2+}]_i$ in sensory neurons ($15.96 \pm 4.24\%$, 487 neurons, n=6), with a peak within 5-15s (Supplementary Figure 3A,C). Such increase was not observed in sensory neurons ($6.56 \pm 1.34\%$, 396 neurons, n=5) of TRPV4-deficient mice (Supplementary Figure 3A, D) or in sensory neurons pre-treated with the TRPV4 antagonist HC067047 (Supplementary Figure 3B). A significant increase in the number of neurons responding to 5,6-EET (10 μ M) was observed in neurons projecting from the colon (FB positive) compared to the total population or to FB negative sensory neurons (Figure 4A) as previously described for TRPV4 expression¹⁶. Stimulation of FB-labelled neurons with 5,6-EET but not with its metabolites 5,6-DHET induced calcium mobilization in a dose-dependent manner (Figure 4B). Pretreatment of these sensory neurons with a TRPV4 antagonist (HC067047) prevented the calcium influx induced by 5,6-EET (10 μ M), demonstrating that 5,6-EET signals through TRPV4 activation in these colon-projecting sensory neurons (Figure 4B). Exposure of FB-labeled neurons to lipids extracted from biopsies of IBS-D and IBS-C patients induced a significant increase in calcium mobilization, compared to lipids from biopsies of HC (Figure 4C). In contrast, exposure of those neurons to lipids from biopsies of IBS-M patients had no

effect on calcium mobilization (Figure 4C). Pretreatment of neurons with a TRPV4 antagonist (HC067047, 10 μ M) significantly inhibited the calcium influx induced by neurons exposure to lipid extracted from biopsy of IBS-D patients, but not from IBS-C patients (Figure 4C). Moreover, exposure of those neurons to IBS-D patient biopsy lipids from which EETs have been retained had no effect on calcium mobilization (Figure 4C). In the same manner, exposure of FB-labeled neurons to lipids extracted from colons of hypersensitive mice, but not from normo-sensitive mice, induced a significant increase in calcium mobilization (Figure 4D). This increase was inhibited by a pretreatment with a TRPV4 antagonist (HC067047, 10 μ M) (Figure 4D). In human DRG T12, TRPV-4 immunoreactivity was observed in 35% of neurons, which were labeled by the pan-neuronal marker PGP9.5 (Figure 4E). The staining was predominant in neurons with a diameter from 10 to 50 μ M (Figure 4F).

IBS-D tissue supernatants caused visceral hypersensitivity in mice in a TRPV4-dependent manner

In a previous study, we have determined that 3-days after inter-vertebral injection of TRPV4-targeted siRNA, the inhibition of TRPV4 expression in DRG sensory neurons was maximal⁴. Therefore, we used the same time-point of 3 days after inter-vertebral injection of TRPV4- or mismatched siRNA for the following functional studies. The allodynia and hyperalgesia observed 3 hours after intracolonic administration of supernatants from IBS-D patient were prevented by an inter-vertebral injection of TRPV4-targeted siRNA (Figure 5). In contrast, inter-vertebral injection of the mismatch siRNA had no effect on hyperalgesia and allodynia induced by intracolonic administration of supernatant from IBS-D patient (Figure 5). As previously described³, we confirmed that intracolonic administration of supernatants from control patient did not induce hypersensitivity in response to colorectal distension (Figure 3).

Sensory neurons exposed to IBS-D patient biopsy supernatants released a TRPV4 agonist in a protease-, PAR₂- and CYPe dependent manner.

We examined the effects of supernatants from human colonic biopsies on the quantity of 5,6-EET released in cultured mouse DRG neurons. While 5,6-EET was detected and significantly increased in tissues from IBS patients, it was never detected in supernatants from incubated biopsies (Supplementary Figure 2), suggesting that this mediator stays trapped within colonic tissues. When neurons are exposed to biopsy supernatants from IBS-D patients, a prompt increase in 5,6-EET was observed, compared to neurons exposed to supernatants from biopsies of HC (Figure 6A). Since the level of 5,6-EET was undetectable in biopsy supernatants, it can be concluded that the release of 5,6-EET observed under those experimental conditions, comes from stimulated neurons. We further investigated the nature of this stimulation, by adding to IBS-D biopsy supernatants, a large spectrum serine-protease inhibitor: AEBSF. The addition of AEBSF to supernatants from IBS-D biopsies completely abolished the synthesis of 5,6-EET by DRG neurons (Figure 6A). This suggests that serine protease activity in IBS-D biopsy supernatants is responsible for the activation of sensory neurons and their further synthesis of 5,6-EET. We investigated whether this serine protease-induced release of 5,6-EET by sensory neurons was dependent on PAR₂ activation, by repeating the experiment in neurons isolated from mice deficient for PAR₂ (PAR₂^{-/-}). No increase in 5,6-EET was observed in PAR₂^{-/-} DRG neurons after they have been exposed to supernatants from IBS-D patients (Figure 6A). In addition, we observed that neurons incubated with a PAR₂-agonist peptide (PAR₂-AP; SLIGRL, 100 μM), but not with the control PAR₂-inverse peptide (PAR₂-IP; LRGILS, 100 μM) produced significantly higher amounts of 5,6-EET, compared to neurons incubated with vehicle (Figure 6B). To determine by which pathway PAR₂ activation induced 5,6-EET production, neurons were pretreated with an inhibitor of cytochrome epoxygenase (CYPe; 17-ODYA, 0.1mM), an inhibitor

lipxygenase (LOX; NDGA, 0.1mM) or an inhibitor of COX (indomethacin, 0.1mM) (Supplementary Figure 1). Pretreatment of sensory neurons by NDGA or indomethacin had no effect on the production of 5,6-EET by sensory neurons treated with the PAR₂-AP (Figure 6B). In contrast, 17-ODYA pretreatment decreased the production of 5,6-EET by sensory neurons treated with IBS-D supernatant or with the PAR₂ agonist (Figure 6A, B). Taken together, these results show that proteases released by colonic tissues from IBS-D patients can increase the synthesis of 5,6-EET in sensory neurons, by activating PAR₂ and CYPe, independently of LOX or COX pathways. In neurons projecting from the colon, we evaluated the potentiation of TRPV4 by PAR₂ activation. Inhibition of CYPe or SRC kinase decreased PAR₂-induced potentiation of Ca²⁺ responses to TRPV4 agonist (Figure 6C). This result identifies SRC kinase and CYPe as the two mechanisms linking PAR₂ to TRPV4 activation in colonic sensory neurons.

Discussion

Our results show that: 1) a PUFA metabolite agonist of TRPV4 (5,6-EET) is increased in biopsies from patients with IBS-D compared to control; 2) the concentration of 5,6-EET in biopsies is correlated to pain and bloating severity and frequency scores 3) PUFA metabolite agonists of TRPV1 and TRPA1 are not increased in biopsies of IBS patients, compared to HC; 4) PUFA metabolites extracted from IBS-D and IBS-C patient tissues or hypersensitive mouse colonic tissues can signal to sensory neurons; 5) PUFA metabolites from biopsies of IBS-D patients signal through a mechanism involving TRPV4 activation; 6) supernatant from IBS-D patient tissues, when administrated into the colon of mice, causes visceral hypersensitivity through a TRPV4-dependent mechanism; 7) TRPV4 is expressed in human DRG neurons; and 8) protease activity released from IBS-D patients biopsies induces, by activating PAR₂ and downstream CYPe in sensory neurons, the production of PUFA metabolites. Taken together, these data clearly highlight the potential role of TRPV4 and of its endogenous agonist 5,6-EET in hypersensitivity associated IBS-D.

Although basic science results point to a crucial role for TRP channels in general pain responses, there is a severe lack of knowledge concerning the nature of the endogenous agonists released in human pain-associated diseases, and that could activate those channels. Watanabe and co-workers have demonstrated that AA activates TRPV4 in an indirect way, involving the CYPe-dependent formation of EET¹⁰. Which type of TRP channel can be activated *in vivo* by 5,6-EET in sensory neurons is still under debate. Indeed, a recent study has shown that 5,6-EET activates TRPA1 instead of TRPV4 in sensory neurons¹⁷. This is in contrast with our present results, where we have observed that 5,6-EET is able to activate TRPV4 on sensory neurons. The discrepancy between both studies could be explained by the type of sensory neurons that were considered. While the first study considered all neurons

present in the DRG, in our study we have focused our recordings on sensory neurons labeled by fast blue injected into the colon, thereby discriminating sensory neurons projecting from this tissue. In our study we quantified a significant increase of fast blue neurons responding to 5,6-EET compared to the total population of sensory neurons. In this tissue-specific population of neurons, 70% of cells are positive for TRPV4 mRNA expression, as determined by *in situ* hybridization, whereas in the general population of sensory neurons, positive staining for TRPV4 is under 20%¹⁶. Moreover, the mechanosensory responses of colonic serosal and mesenteric afferents are enhanced by 5,6-EET only in wild-type mice and are reduced in TRPV4^{-/-}¹⁶. These results are consistent with our study showing that a decrease of TRPV4 expression in sensory neurons inhibits the hypersensitivity induced by intracolonic administration of supernatants from IBS patient biopsies. Moreover, we observe here that the quantity of 5,6-EET in biopsies correlates with the pain severity and frequency scores. Taken together, these results reinforce the concept that TRPV4, which is expressed in human DRG neurons, and its agonist, 5,6-EET, are selectively implicated in visceral sensation. We further propose here that TRPV4 could represent an important receptor mediating hypersensitivity symptoms associated with IBS, and that among other TRPs, TRPV4 appears as a privileged receptor activated in IBS states. It is important to note though that there might be interspecies differences. In the present approach, we investigate the effects of human mediators on mouse neurons and VMR. However, we confirmed that like in mice, human primary afferents, express TRPV4.

In a previous study, we have established that proteases released by tissues from IBS patients can directly stimulate sensory neurons and generate hypersensitivity symptoms through the activation of PAR₂³. We have also demonstrated that TRPV4 activation is involved in the hypersensitivity induced by PAR₂ activation in mice⁵. The link that seems to exist between proteases, PAR₂ and TRPV4 has been further demonstrated by a recent study

presenting a functional coupling between PAR₂ and TRPV4¹⁸. This coupling, identified in a model of HEK cell line and in primary nociceptive neurons, involves the generation of a yet to be defined AA-derived TRPV4 activator and a tyrosine kinase signaling pathway¹⁸. Whereas in HEK cells, PAR₂-AP provokes a transient increase in intracellular calcium, in HEK-TRPV4 cells, the agonist peptide induces a similar rapid increase in intracellular calcium concentration that is markedly sustained overtime¹⁸. This sustained response is decreased by an inhibitor of CYPe, suggesting that PAR₂ activation could induce the metabolism of AA into EET leading to TRPV4 activation¹⁸. We have now demonstrated that indeed, PAR₂-AP is able to induce 5,6-EET synthesis in sensory neurons. Moreover, we have highlighted the link between proteases released by biopsies from IBS-D patients and the increase in 5,6-EET synthesis. We have established that proteases released by biopsies of IBS-D patients induce an increase in 5,6-EET concentration in sensory neurons, through the activation of PAR₂ and CYPe. Since we have established that the effects of serotonin and histamine on visceral sensitivity are dependent on TRPV4 activation⁵, it can be hypothesized that the production of 5,6-EET by sensory neurons is not restricted to PAR₂ activation, but might occur following serotonin or histamine treatment as well. Taken together these data showed that soluble mediators secreted by biopsies from IBS patients can activate CYPe that metabolizes AA into 5,6-EET, which could then be responsible for TRPV4 activation. Nevertheless, several studies have established that PAR₂ agonists induce TRPV4 phosphorylation, which participates in the activation of the receptor^{4, 18-20}. Recently, Dr. McIntyre's group has established that PAR₂-mediated receptor-operated gating of TRPV4 was dependent of tyrosine kinases and independent of G α_q stimulation²⁰. In our study focused on sensory neurons projecting from the colon, inhibition of both pathways, CYPe and kinase, was able to decrease the activation of TRPV4 by PAR₂ agonist. Thereby, it can be concluded that both phosphorylation of TRPV4 and CYPe-induced production of TRPV4 endogenous

agonists are important in IBS-associated TRPV4 activation. As proteases and TRPV4 seem to be implicated in inflammatory diseases such as inflammatory bowel diseases, arthritis or edema, the production of endogenous TRPV4 agonists could also be increased in inflammatory conditions^{6, 21-24}. Thus, not only in IBS but also in other pathophysiological conditions, the characterization of TRP channel endogenous agonists and the signaling events leading to their production or to TRP phosphorylation may highlight novel therapeutic targets. Upon consideration of such targets, a therapeutic aim could be to decrease the sensitization of TRP channels, without affecting their basal response.

In a recent study, authors have performed a microarray analysis of colonic tissues in stressed animals compared to controls²⁵. As a first step they confirmed the increase of visceral sensitivity in maternally-separated animals. Then, they demonstrated an increase of PTGS2 gene expression coding for COX-2 enzyme in hypersensitive animals²⁵. This enzyme metabolizes AA into PGE₂; therefore up-regulation of this gene could explain the increase of PGE₂ quantified in this model²⁶. Interestingly, they have quantified an increase in gene expression of TRPV4 and a decrease in gene expression of TRPA1²⁵. This last result strengthened the concept that TRPV4 is a major actor of visceral hypersensitivity. The increase of PGE₂ has also been reported in plasma of IBS-D patients²⁷ and in supernatants from IBS biopsies². We have quantified here PGE₂ in biopsies from IBS-D patients, confirming the increase observed in these other studies. It is well established that PGE₂ can cause an increased excitability of primary sensory fibers²⁸. Moreover, several PUFA metabolites among which thromboxane A₂, PGE₂, LtB₄ or PGD₂, have been studied for their capacity to regulate visceral afferent fibers²⁹⁻³². In agreement with those results, we demonstrated that PGE₂ quantity in biopsies correlated with pain frequency score. The increase of PGE₂ that we have observed in IBS-D biopsies could sensitize directly nerve fibers or TRPV4 activation by 5,6-EET³³. In contrast to PGE₂, we have observed that PGA₁ is

decreased in biopsies from IBS-D patients. While increase in 5,6-EET and PGE₂ was observed only in IBS-D subgroup, the decrease in PGA₁ was observed both in IBS-D and in IBS-C patients, compared to HC. Increase in inflammatory mediators such as cytokines or PGE₂ has been mostly observed in IBS-D and not in other subgroups³⁴. The fact that PGA₁ was also decreased in IBS-C and that PUFA metabolites extracted from biopsies of IBS-C induced calcium flux in sensory neurons independently of TRPV4 opens a new area of research for the characterization of mediators specific to this subgroup.

Taken together, results presented in this study highlighted the potential role of TRPV4 and of its endogenous agonist in visceral pain associated with IBS-D symptoms. We demonstrate that increase in proteolytic activity in IBS-D patients induces the production of 5,6-EET and the activation of TRPV4 in sensory neurons. The characterization of endogenous agonists of TRPs channels that are overproduced in disease could represent novel therapeutic opportunities to reduce channel activation.

Figure 1: PUFA metabolites quantification in biopsies from IBS patients and HC.

TRPV4 agonists (**A**; 5,6-EET and 8,9-EET), TRPV1 agonist (**B**; LTB₄, 5-HETE, 12-HETE and 15-HETE), TRPA1 agonists (**C**; PGA₁, 15dPGJ₂ and 8-isoPGA₂), resolvins precursors (**D**; 17-HDoHE and 18-HEPE) and PGE₂ (**E**) were quantified in biopsies from HC (white circles), IBS patients (Diarrhea-predominant: D, gray circles; Constipation-predominant: C, gray triangles; or Mixed: M, gray squares). Data are represented as scattered dot plot with line at mean. * significantly different from HC, p<0.05 (*), p<0.01 (**).

Figure 2: Correlation between the concentration of 5,6-EET or PGE₂ in biopsies from IBS patients and HC and abdominal pain and bloating scores.

Abdominal pain severity and frequency scores and bloating severity and frequency scores were determined for each individual (black circle). Coefficient of correlation (r) between the scores and the concentration of 5,6-EET (**A**) or PGE₂ (**B**) in biopsies from IBS patients and HC was calculated. Data are represented as aligned dot plot with mean and errors linear regression lines. The significance of each correlation is indicated on each graphic by the p value.

Figure 3: PUFA metabolites quantification in colon of mice treated by human colonic biopsy supernatants from IBS-D patient or HC.

(**A**) Mouse visceral motor response (VMR) to increasing pressures of distension in mice before (baseline white square) or 6 hours after intracolonic administration of supernatant from biopsy of IBS-D patients (black triangle) or HC (gray circle). Data are mean ± SEM; n = 11 for the biopsy supernatants from control patients; n = 18 for IBS biopsy supernatants. **P < 0.01, *P < 0.005 compared with baseline. TRPV4 agonists (**B**; 5,6-EET and 8,9-EET), TRPV1 agonists (**C**; LTB₄, 5-HETE, 12-HETE and 15-HETE), TRPA1 agonists (**D**; PGA₁, 15dPGJ₂ and 8-isoPGA₂), resolvins precursor (**E**; 17-HDoHE and 18-HEPE) and PGE₂ (**F**) were quantified in mouse colon treated by supernatant from biopsies of HC (gray bar) or IBS-D patients (black bar). Data are mean ± SEM. * p<0.05, significantly different from control group.

Figure 4: Calcium mobilization in sensory neurons exposed to PUFA metabolites from mouse colon treated by human colonic biopsy supernatants.

(**A**) Percentage of total, fast-blue positive (FB (+)) or Fast-blue-negative (FB (-)) neurons that responded to 5,6-EET (10

μM) by increasing calcium flux. **(B)** Calcium flux in fast blue positive sensory neurons exposed to 5,6-EET (0.1, 1 or 10 μM; black bar), its vehicle (HBSS-DMSO 0.1%, white bar), its hydrolyzed metabolite 5,6-DHET (10 μM; striped bar) or pre-incubated with a TRPV4 antagonist (HC067047, 10 μM) and treated with 5,6-EET (10 μM; gray bar). **(C)** Calcium flux in fast blue positive sensory neurons exposed to PUFA metabolites from biopsy of HC (white bar), IBS patients (black bar) pre-incubated or not with a TRPV4 antagonist (HC067046, 10 μM; gray bar) or IBS patients in which EET have been removed (striped bar). **(D)** Calcium flux in fast blue positive sensory neurons exposed to HBSS-DMSO 0.1% or PUFA metabolites from colon of mouse treated by supernatant of biopsies from HC (gray bar) or IBS patients (black bar) pre-incubated or not with a TRPV4 antagonist (HC067047, 10 μM). Data are mean ± SEM. * significantly different from control group, p<0.05 (*), p<0.01 (**), p<0.001 (***); # p<0.05, significantly different from corresponding treated group (**A**, **C**, **D**), ### p<0.001, significantly different from the total/5,6-EET group (**B**). **(E)** Expression of TRPV4 in human sensory neurons. TRPV4 (in green and indicated by an arrow) and PGP9.5 (in red) expression in human DRG T12; average of the percentage of TRPV4-immunoreactive (white) and non-immunoreactive neurons (black) of 3 different T12 DRGs. **(F)** Distribution of TRPV4-immunoreactive human neurons according to the diameter of the perikarya.

Figure 5: Mouse visceral sensitivity in response to human colonic biopsy supernatants. Differences in visceromotor response (VMR) to increasing pressures of colorectal distension before, and 3 hours after the intracolonic administration of biopsy supernatants from IBS patients (striped and black bars), or control patients (white and gray bars). Mice received an inter-vertebral injection of siRNA control (striped and white bars), or TRPV4-directed siRNA (gray and black bars) 3 days before colorectal distension experiment. Data are mean ± SEM. * significantly different from control supernatant groups, p<0.01 (**), p<0.001 (***); # significantly different from the group SiRNA TRPV4 + IBS supernatant, p<0.01 (##), p<0.001 (###).

Figure 6: 5,6-EET quantification in mouse sensory neurons treated by human colonic biopsy supernatants from IBS-D patient or HC, a PAR₂ mechanism. 5,6-EET quantification in sensory neurons exposed to **(A)** HBSS (squared bar) or biopsy supernatants from HC (striped bar), or IBS patients (black bars). Treatment with biopsy supernatant has been performed in WT or PAR₂ deficient (PAR₂^{-/-}) mice and with or without a protease inhibitor (AEBSF, 0.2 mM) or CYPe inhibitor (17-ODYA, 0.1mM). 5,6-EET quantification in sensory neurons exposed to PAR₂-IP (LRGILS, 100 μM; white bar), PAR₂-AP (SLIGRL,

100 μ M; gray bar). Treatment with PAR₂-AP has been performed with or without LOX inhibitor (Nordihydroguaiaretic Acid, NDGA, 0.1mM), COX inhibitor (Indomethacin, Indo, 0.1mM) or CYPe inhibitor (17-ODYA, 0.1mM). (C) Calcium influx mediated by GSK1016790A (50nM) in Fast Blue–positive DRG neurons innervating the mouse colon pretreated with or without SLIGRL (100 μ M), 17-ODYA (0.1mM) or Src1 (10 μ M). Data are mean \pm SEM. *p<0.05 significantly different from the corresponding control group, #, significantly different from IBS group (A, B) or from SLIGRL/GSK1016790A group (C), p<0.05 (#), p<0.01(##). (D) Hypothesized mechanism of PUFA metabolites-induced hypersensitivity in IBS-D patients. Proteases released by IBS-D patient biopsy activate PAR₂ on the terminals of sensory neurons. PAR₂ activates cytochrome P450 epoxygenase (P450) which metabolizes arachidonic acid (AA) in 5,6-EET. This TRPV4 agonist then promotes TRPV4 channel opening and influx of extracellular Ca²⁺ leading to neuronal activation and hypersensitivity.

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Author names in bold designate shared co-authorship

Table 1: Characteristics of patients from which biopsies were collected for PUFA metabolites quantification and for culture media incubations

	Biopsies for: PUFA metabolites quantification		Culture media incubations	
	Control	IBS	Control	IBS
n	11	40	11	20
Age	49 (20-76)	41 (20-69)	49 (20-76)	43 (22-74)
Sex (F/M)	5/6	24/16	5/6	9/11
Bowel habit:				
Diarrhea	0	20	0	20
Constipation	0	10	0	0
Mixed	0	10	0	0

Figure 1

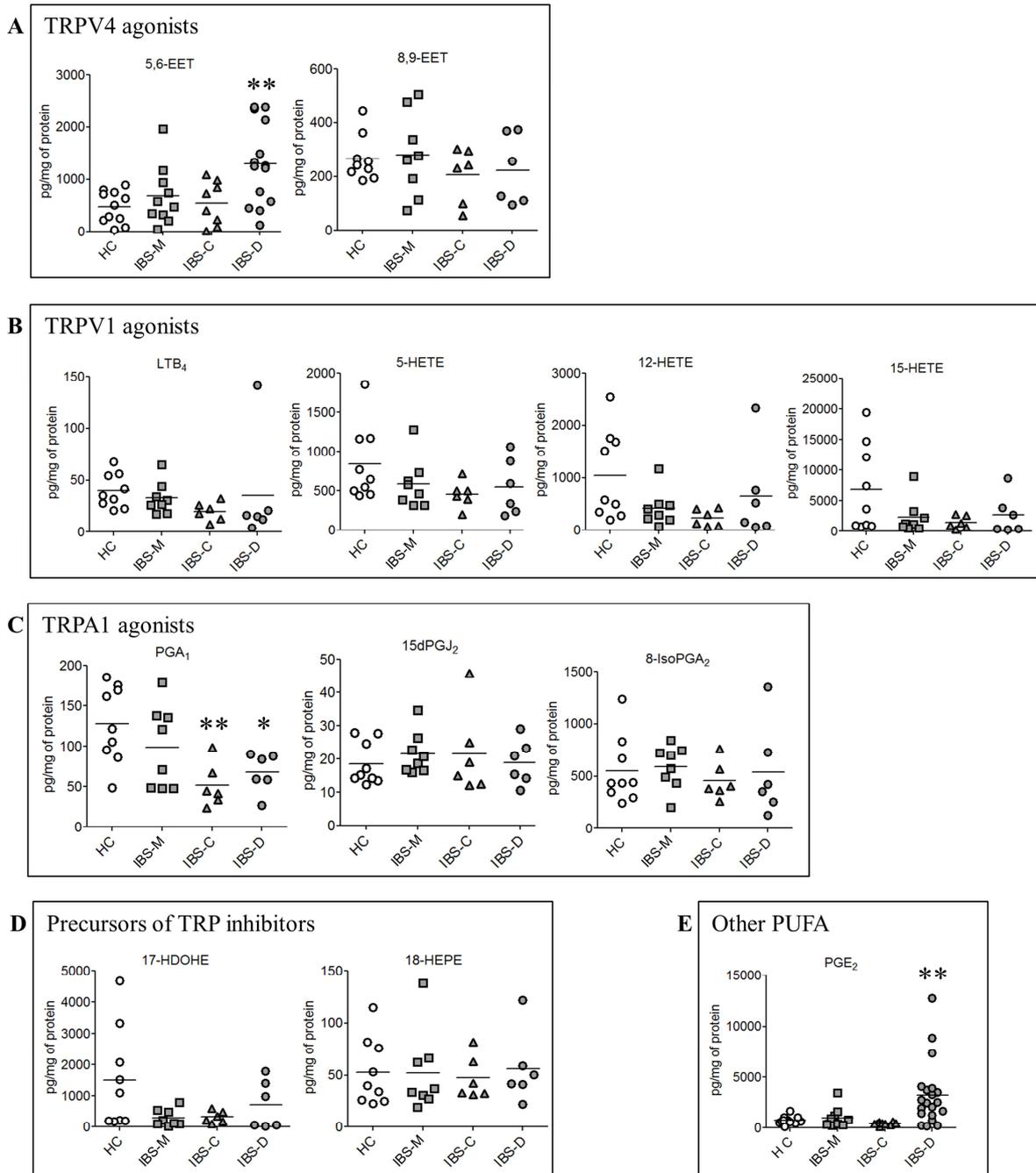


Figure 2

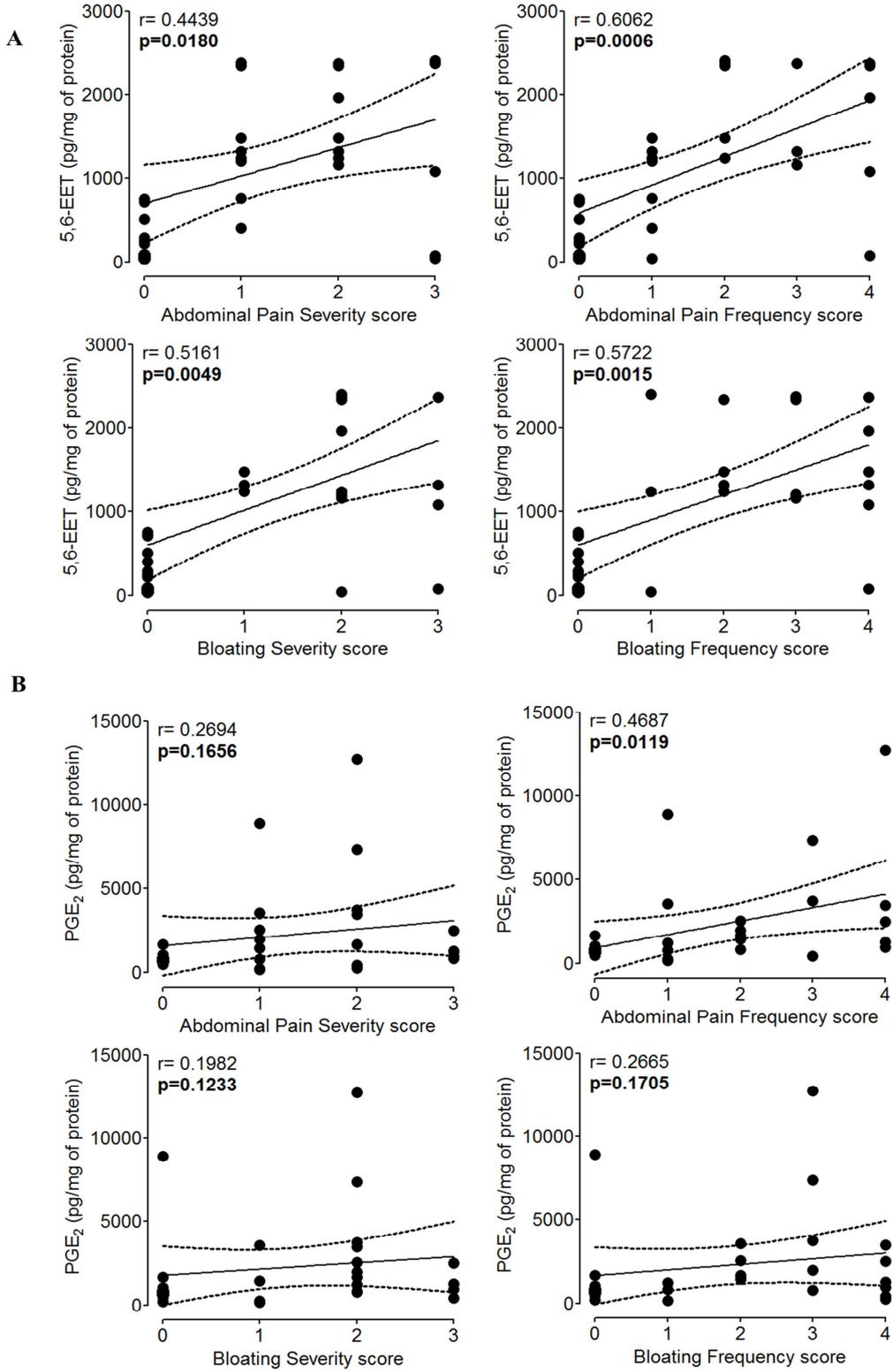


Figure 3

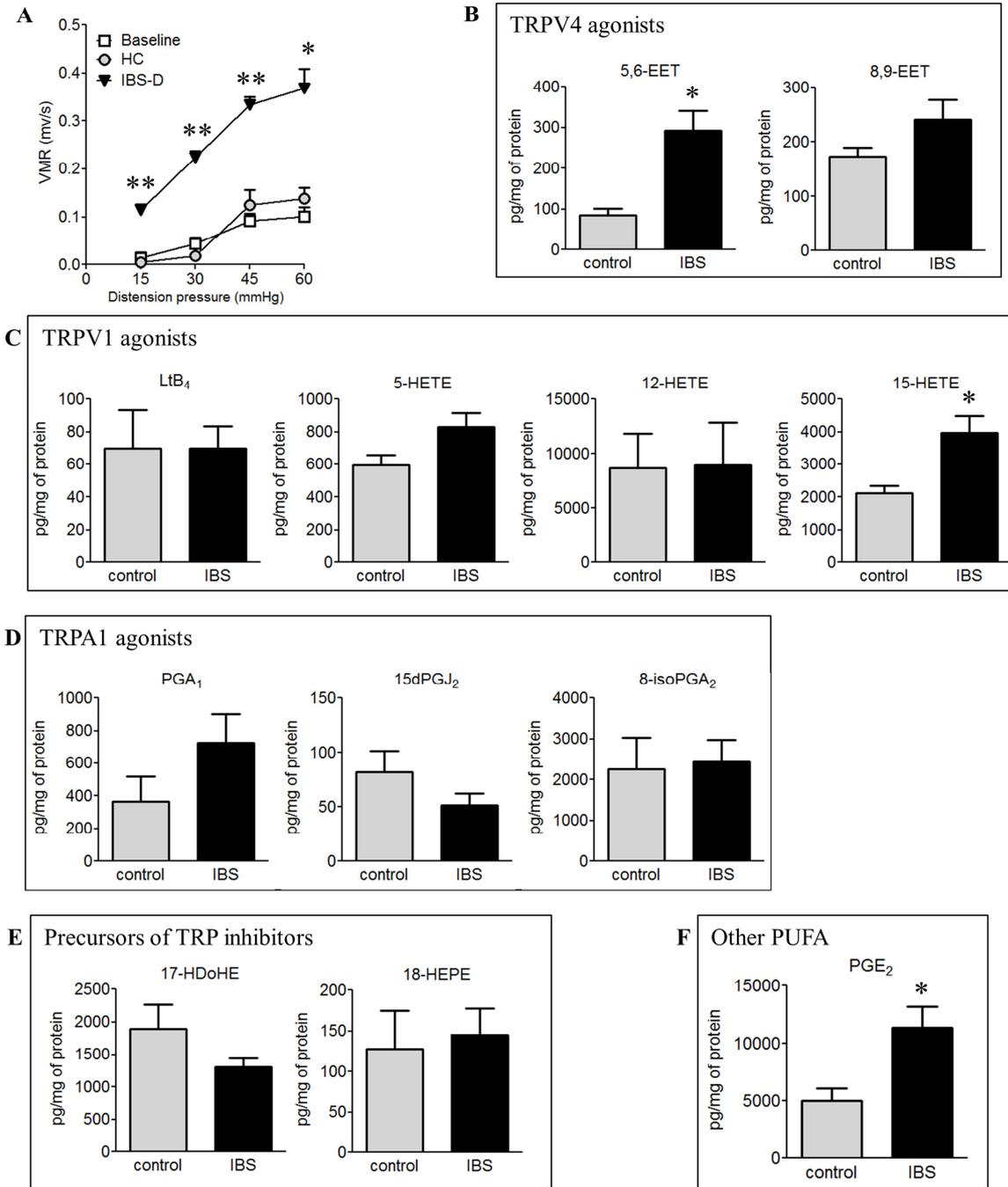


Figure 4

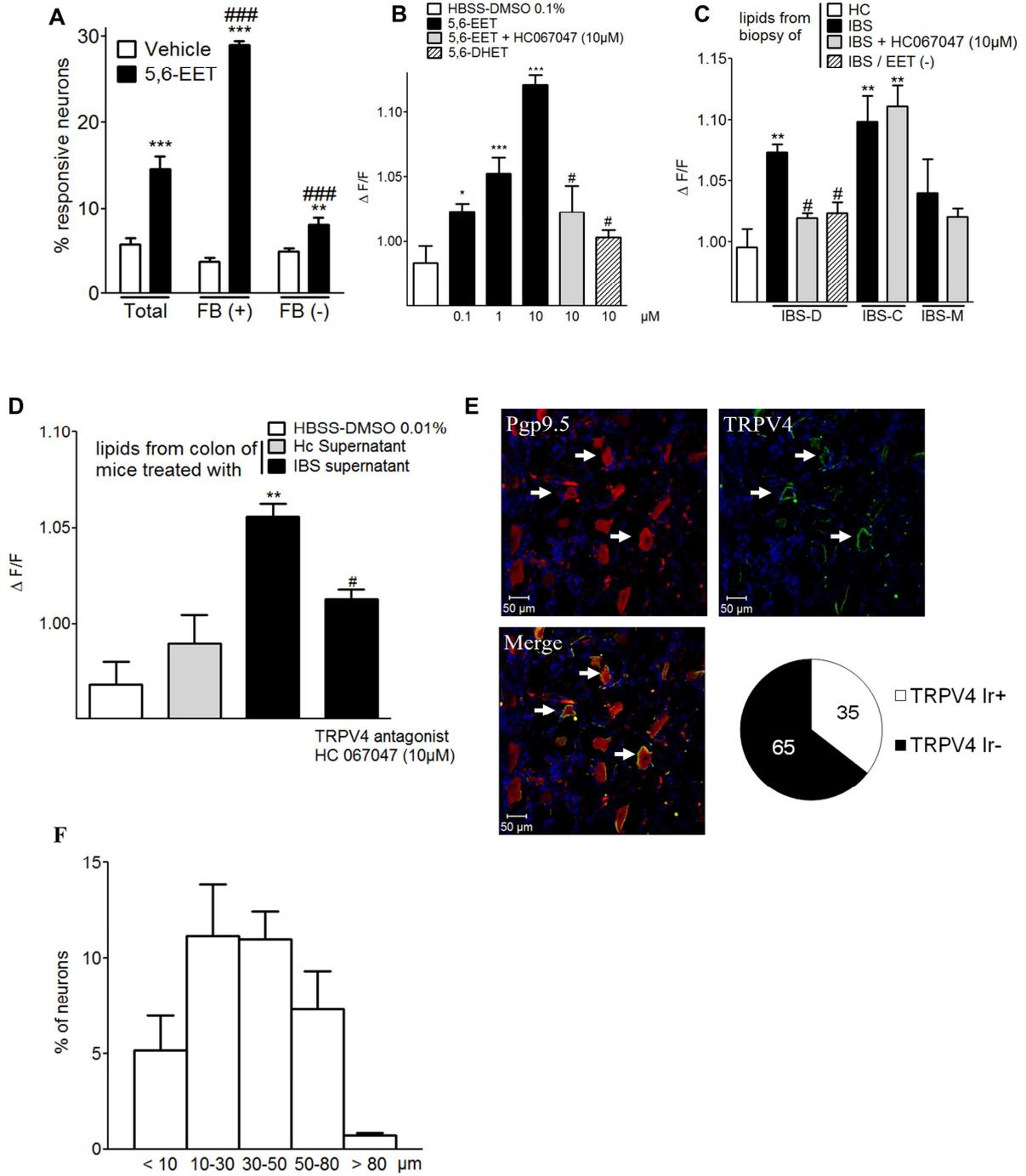


Figure 5

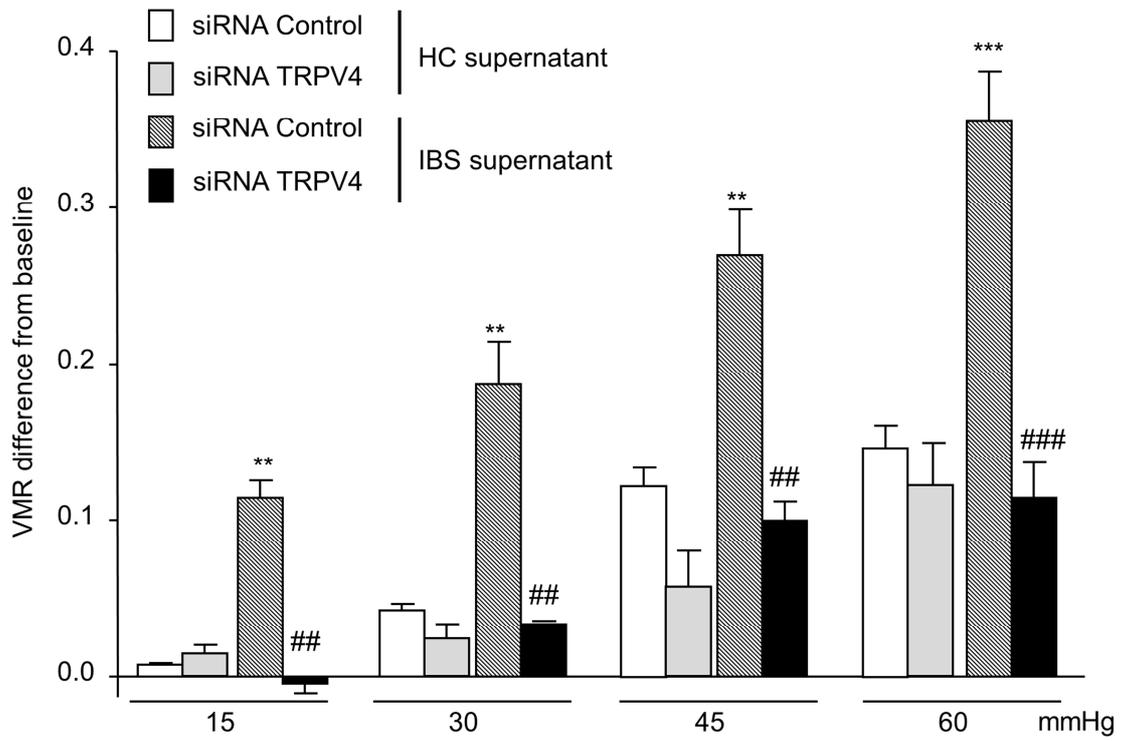
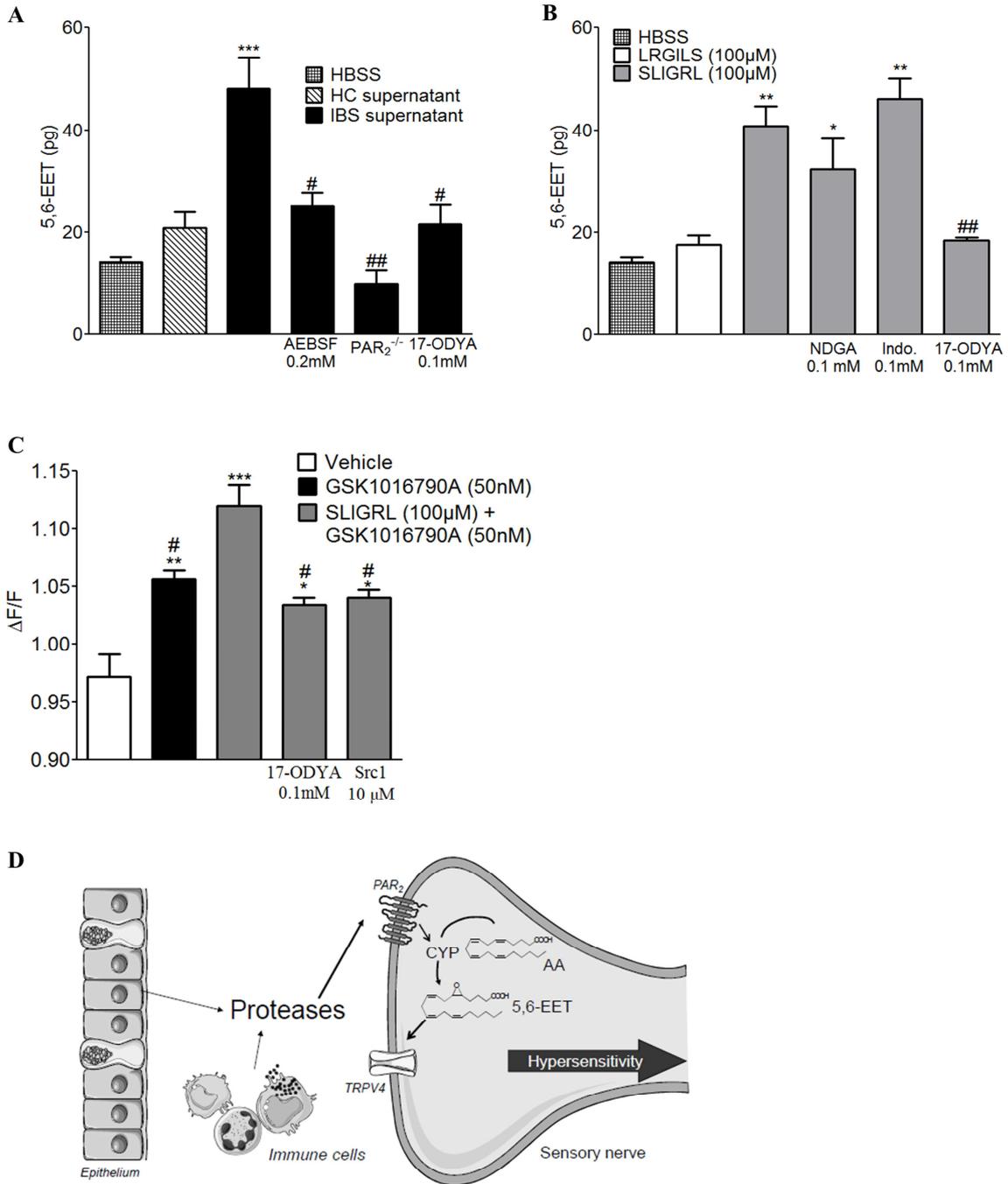


Figure 6



Supplementary method

1. Chemicals

Prostaglandin E2 (PGE₂), PGA₁, 8-iso-PGA₂, 15d-PGJ₂, lipoxin A4 deuterated (LxA₄-d₅), LTB₄, LTB₄-d₄, 15-HETE, 12-HETE, 5-HETE, 5-HETE-d₈, 8,9-EET, 5,6-EET, 18-hydroxyeicosapentaenoic acid (18-HEPE), 17-hydroxy-docosahexaenoic acid (17-HDoHE), RvD1 and RvD2, 5,6-dihydroxyeicosatrienoic acid (5,6-DHET) and nordihydroguaiaretic acid (NDGA) were purchased from Cayman Chemicals (Interchim, Montluçon, FRANCE). Methanol (MeOH), Hank's Balanced Salt Solution (HBSS), indomethacin and 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF) were from Sigma-Aldrich (Saint Quentin Fallavier, France). TRPV4 antagonist, HC 067047, and 17-Octadecynoic Acid (17-ODYA) were from Tocris bioscience (R&D Systems Europe, Lille, France)

2. Animals

C57Bl6 male mice (6-8 weeks) were purchased from Janvier (Le Genest Saint Isle, France), and PAR₂-deficient mice (PAR₂^{-/-}) originally from Jackson laboratory (Bar Harbor, USA), were bred at CREFE Animal Care facility (Toulouse, France). All procedures were approved by institutional Animal Care Committees (MP/01/64/09/12).

3. DRG neurons isolation

Dorsal root ganglia from T10-L1 and L6-S1 were rinsed in cold HBSS (Invitrogen), and enzymatically dissociated as described previously¹. Neurons were plated in 6 wells (fluorescence Greiner bio one, Dominique Dutscher, Brumath, France) and cultured for 24 hours¹. In a first set of experiments, neurons of wild type and PAR₂ deficient mice were treated with supernatant of IBS or control patients and pretreated (30 min before) with an

inhibitor of proteases (AEBSF, 0.2 mM), a CYPe inhibitor (17-ODYA, 0.1mM) or their vehicle (culture medium). In a second set of experiments, neurons were treated with the PAR₂-AP (SLIGRL; 100 μM) or with its control peptide for 2 hours and pretreated (30 min before) with a CYPe inhibitor (17-ODYA, 0.1mM), a COX inhibitor (indomethacin, 0.1mM), a LOX inhibitor (NDGA, 0.1 mM) or their vehicle (culture medium). After those different treatments 5,6-EET was quantified.

4. Lipid extraction

Mouse Colons, human biopsies, supernatant of biopsy or mouse sensory neurons were crushed with a FastPrep®-24 Instrument (MP biomedical) in 500 μL of HBSS (Invitrogen) and 5 μL of internal standard mixture (400 ng/mL). After centrifugation, supernatants were collected and submitted to solid-phase extraction using HRX-50 mg 96-well (Macherey Nagel, Hoerd, France)². Briefly, plates were conditioned with 2 mL of MeOH and 2 mL of H₂O/MeOH (90:10, v/v). The sample was loaded at flow rate of about 1 drop per 2 s. After complete loading, columns were washed with 2 mL of H₂O/MeOH (90:10, v/v). After drying under aspiration, lipid mediators were eluted with MeOH (2 mL) to elute all of PUFA metabolite or with H₂O/MeOH (40:60, v/v) to do not eluted EET (sup. Table 1). Solvent was evaporated under N₂ and samples were with MeOH and stored at -80 °C for liquid chromatography/tandem mass spectrometry (LC-MS/MS) measurements or dissolved HBSS/DMSO 0.1% for calcium signaling experiments.

5. Liquid chromatography/tandem mass spectrometry measurements

By this technique we performed the quantification of PGE₂, 5-, 6-, 8-, 9-EET, PGA₁, 8-iso PGA₂, 15dPGJ₂, LTB₄, 5-, 12-, 15-HETE, 18-HEPE, 17-HDoHE, RvD1 and RvD2 in

human or mouse intestinal tissue as previously describe². To simultaneously separate 14 lipids of interest and 3 deuterated internal standards (LxA₄-d₅, LTB₄-d₄, 5-HETE-d₈), LC-MS/MS analysis was performed on HPLC system (Agilent LC1290 Infinity, Les Ulis, France) coupled to Agilent 6460 triple quadrupole MS (Agilent Technologies) equipped with electro-spray ionization operating in negative mode. Reverse-phase HPLC was performed using ZorBAX SB-C18 column (2.1 mm, 50 mm, 1.8 μm) (Agilent Technologies) with a gradient elution. Compounds were separated and quantified as previously describe².

6. Calcium imaging of mouse sensory neurons

Seven days after fast-blue (FB) injection³, DRG were dissociated and plated in 96 wells (fluorescence Greiner bio one, Dominique Dutscher, Brumath, France) for calcium signaling assay¹. In a first set of experiment neurons were treated with 5,6-EET (0.1, 1 and 10 μM) or vehicle (HBSS/DMSO 0.1%). In a second set of experiments, neurons were pre-incubated for 5 minutes with a TRPV4 antagonist (HC067047) or vehicle (HBSS/DMSO 0.01%) and treated with 5,6-EET (10 μM) or its vehicle. In a third set of experiments, neurons were treated with lipids extracted from mouse colon treated with supernatant of IBS or control patients or their vehicle (HBSS/DMSO 0.1%). In a last set of experiments, neurons were pre-incubated for 5 minutes with a TRPV4 antagonist (HC067047) or vehicle (HBSS/DMSO 0.01%) and treated with lipids extracted from mouse colon treated with supernatant of IBS-D or control patients or their vehicle.

7. Colorectal distension and electromyography recordings

Mice were administrated intracolonicly with 150 μL of biopsy supernatants from control or IBS-D patients. We performed a session of CRD and recorded visceromotor

responses (VMR) from implanted electrodes before and 3 hours after biopsy sample administration⁴. Data are presented as the difference between the VMR induced by the distension performed before and after intracolonic treatments. Mice also received 3 intervertebral injections between L5-L6 of TRPV4 siRNA or a mismatched siRNA as previously described³. After the last session of distension mouse colons were harvested to perform lipid extraction.

8. Patients

Patients were recruited from outpatient clinics of the Department of Medical and Surgical Sciences of the University of Bologna, Italy and included according to Rome III criteria for IBS⁵. Healthy Controls (HC) were asymptomatic subjects undergoing colonoscopy for colorectal cancer screening. In particular, in HC we excluded the presence of the following symptoms in the last 12 months: abdominal discomfort or pain, bloating, and bowel habit changes. Exclusion criteria for both IBS and controls included major abdominal surgery, any organic syndrome, celiac disease (excluded by detection of anti-transglutaminase and anti-endomysial antibodies), asthma, food allergy or other allergic disorders. None of these patients or healthy controls was taking non-steroidal anti-inflammatory drugs or other anti-inflammatory drugs (including steroids, anti-histaminics and mast cell stabilizers). Patients and HC gave written informed consent. The study protocol was approved by the local Ethic Committee (64/2004/O/Sper and EM14/2006/O) and conducted in accordance with the Declaration of Helsinki. IBS patients completed a modified Italian version of the Bowel Disease Questionnaire to assess symptoms. Severity and frequency of their symptoms was scored by means of a five-point Likert scale as previously described⁶.

Severity of abdominal pain/discomfort

0 = absence of symptom

1 = non influencing daily activities

2 = diverting from but not inducing modifications of daily activities

3 = causing modification of daily activities

4 = preventing daily activities

Frequency of abdominal pain/discomfort

0 = never

1 = 1 day a week

2 = 2-3 day a week

3 = more than 3 day a week (fewer than 7)

4 = 7 day a week

Severity of bloating

0 = absence of symptom

1 = non influencing daily activities

2 = diverting from but not inducing modifications of daily activities

3 = causing modification of daily activities

4 = preventing daily activities

Frequency of bloating

0 = never

1 = 1 day a week

2 = 2-3 day a week

3 = more than 3 day a week (fewer than 7)

4 = 7 day a week

9. Immunofluorescence in human dorsal root ganglia.

Experiments were conducted under the IRB number IRB00003888,FWA00005831.

Three Human DRG T12 (thoracic position 12) were supplied through the national human tissue resource center from the national disease resource interchange (NDRI). DRG were received unfixed and cryoprotected. DRG were cut into 10 μ m sections in a cryostat (Leica CM1950, Nanterre, France), and mounted on a Superfrost slide (Thermo Fisher Scientific Thermo Scientific, Villebonne-sur-Yvette, France). Slides were washed in phosphate buffered saline (PBS), 0.5% Triton X-100, and 1% bovine serum albumin

(BSA) solution (Sigma, Saint-Quentin Fallavier, France) and incubated overnight at 4°C with the primary antibodies (anti-TRPV4 and anti-PGP9.5 (1:100, AB63003 and 1:100, AB86808, respectively both from Abcam[®], Coges SAS, Paris, France company). After washing, slides were incubated with the appropriate secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 555 washed, and mounted with ProLong Gold reagent containing DAPI (Molecular Probes). Images were acquired using Zeiss LSM-710 confocal microscopes (Carl Zeiss MicroImaging, Jena, Germany) with 20X objective in the inverted configuration. The mean diameter of the neurons were determined using Image J software.

10. Statistical analysis

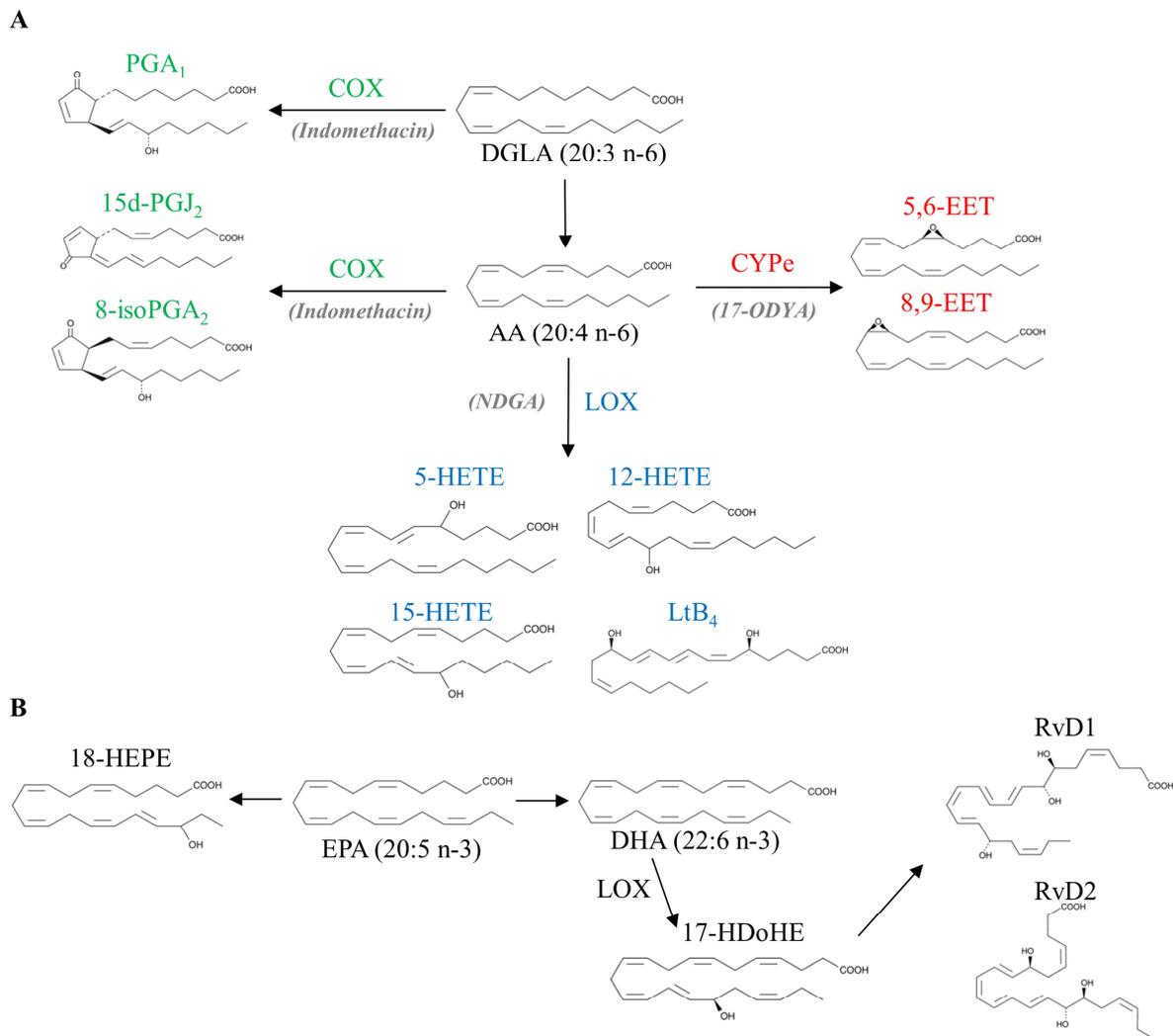
Data are presented as means \pm standard error of the mean (SEM). Analysis used GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Between-group comparisons were performed by unpaired t test. Multiple comparisons within groups were performed by repeated-measures one-way ANOVA, followed by Tukey post-test. Statistical significance was accepted at $P < 0.05$. The coefficient of Correlation r between patient score and PUFA metabolites quantification was calculated using Spearman nonparametric correlation and p values by a two-tailed test.

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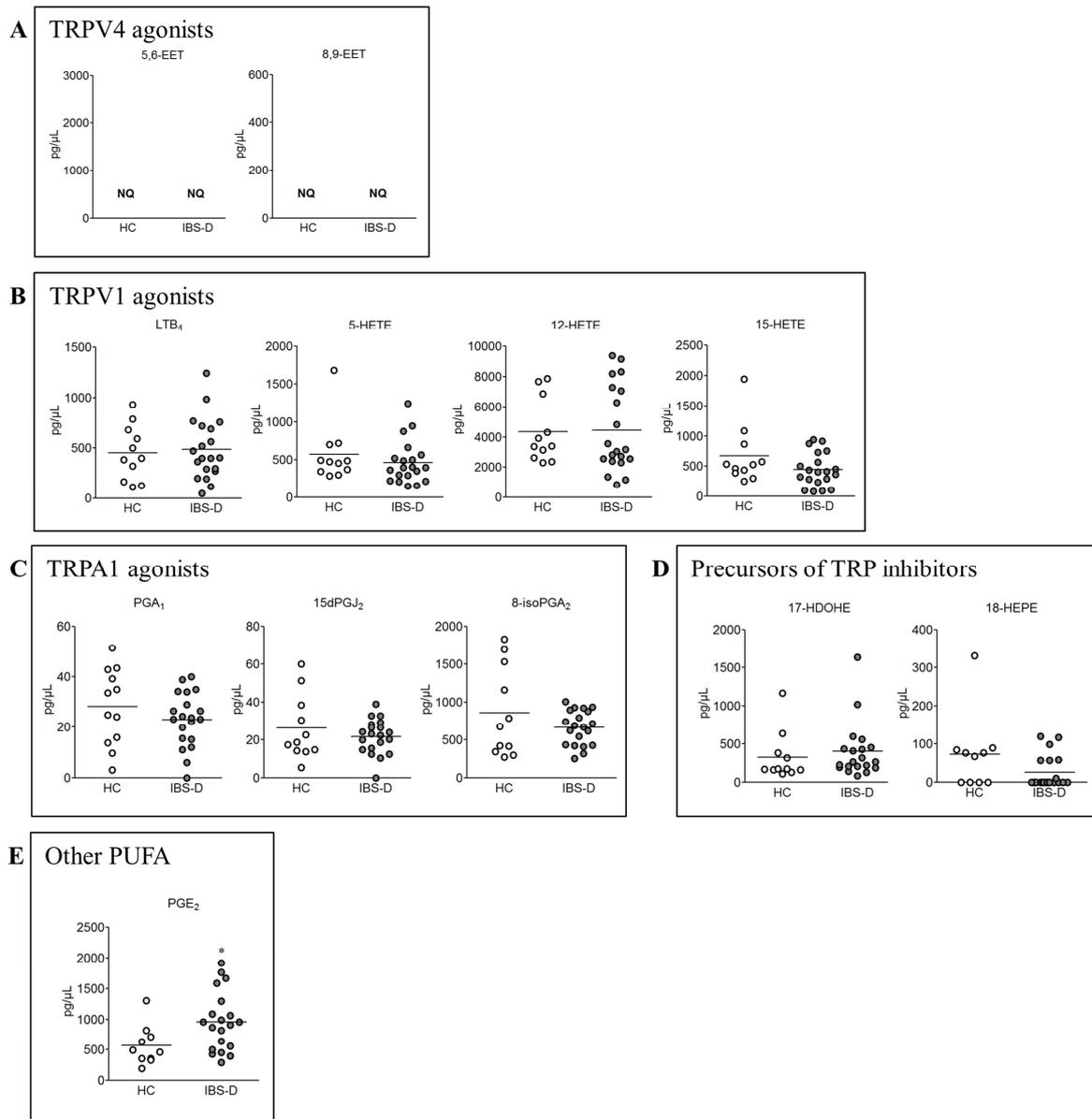
Supplementary Table 1: quantification of PUFA metabolite eluted with two different percentages of MeOH in IBS-D biopsies. ND: not detected

Metabolites	MeOH 100%	MeOH 60%
PGE ₂	3267 ± 702	4652 ± 1002
5, 6-EET	1326 ± 186	ND
8, 9-EET	222 ± 52	ND
PGA ₁	67 ± 10	73 ± 22
8-iso PGA ₂	540 ± 182	488 ± 174
15dPGJ ₂	19 ± 3	29 ± 10
LTB ₄	35 ± 21	47 ± 19
5-HETE	549 ± 147	341 ± 173
12-HETE	652 ± 355	558 ± 278
15-HETE	2704 ± 1329	3004 ± 674



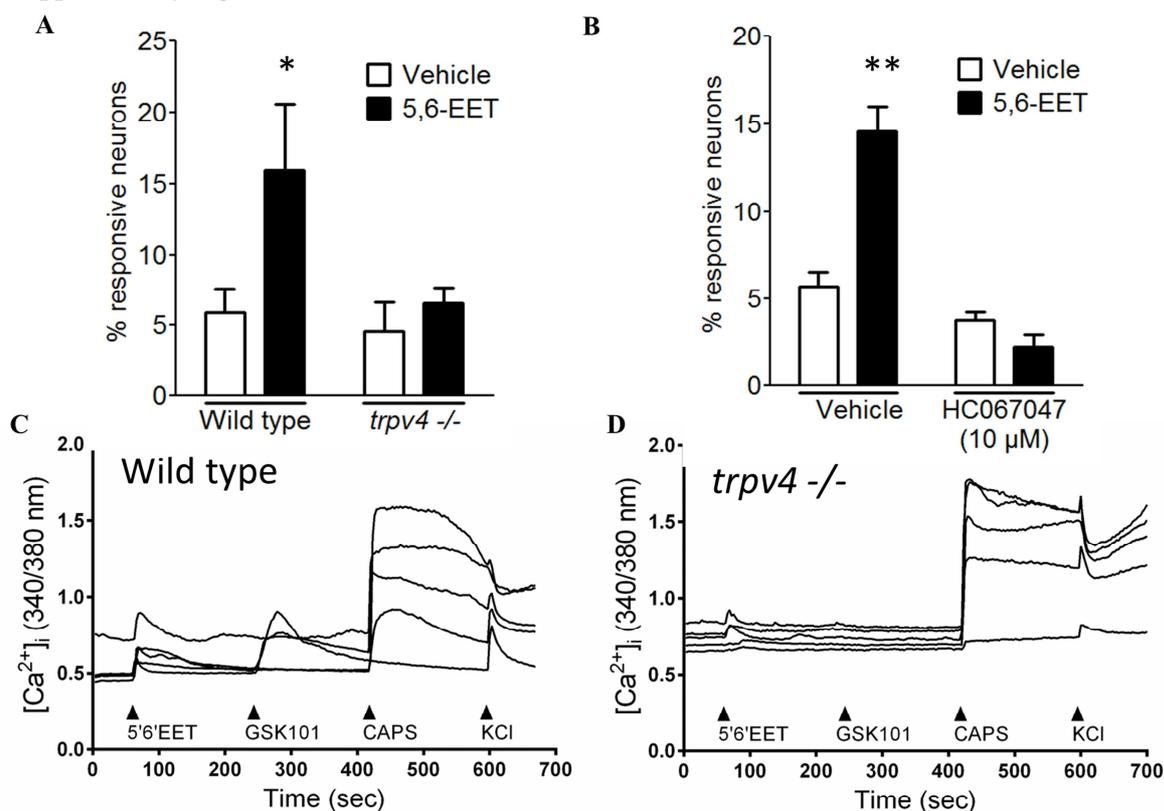
Supplementary Figure 1: Biosynthesis of polyunsaturated fatty acid metabolite agonists (A) and inhibitors (B) of TRP channels. This figure summarizes the endogenous agonists of TRP channels quantified in our study, as well as inhibitors of the different pathways used (*in italics*). **(A)** Metabolism of dihomo- γ -Linolenic acid and arachidonic acid (AA) by cyclooxygenase (COX) pathway produced TRPA1 agonist (in green): prostaglandin A1 (PGA₁), 15-deoxy-prostaglandin J2 (15d-PGJ₂), 8-iso prostaglandin A2 (8-isoPGA₂). Metabolism of arachidonic acid (AA) by lipoxygenase (LOX) pathway produced TRPV1 agonist (in blue): 5-hydroxyeicosatetraenoic acid (5-HETE) and 12-HETE, 15-HETE and leukotriene B4 (LTB₄). Metabolism of arachidonic acid (AA) by cytochrome epoxygenase (CYPe) pathway produced TRPV4 agonist (in red) 5,6-epoxyeicosatrienoic acid (5,6-EET) and 8,9-EET. **(B)** Metabolism of eicosapentaenoic acid (EPA) produced 18-hydroxyeicosapentaenoic acid (18-HEPE, precursor of resolvin E-series) by non-enzymatic oxidation. Metabolism of docosahexaenoic acid (DHA) produced 17-hydroxydocosahexaenoic acid (17-HDoHE), resolvin D1 (RvD1) and RvD2 by LOX pathway.

Supplementary Figure 2



PUFA metabolites quantification in supernatant of biopsies from IBS-D patients and HC. TRPV4 agonists (**A**; 5,6-EET and 8,9-EET), TRPV1 agonist (**B**; LTB₄, 5-HETE, 12-HETE and 15-HETE), TRPA1 agonists (**C**; PGA₁, 15dPGJ₂ and 8-isoPGA₂), resolvins precursors (**D**; 17-HDOHE and 18-HEPE) and PGE₂ (**E**) were quantified in supernatant of biopsies from IIC (white circles), IBS-D patients (Diarrhea-predominant: D, gray circles). Data are represented as scattered dot plot with line at mean. * significantly different from HC, $p < 0.05$ (*). **NQ**: Not quantifiable

Supplementary Figure 3



5,6-EET activates TRPV4 in mouse sensory neurons. Dorsal root ganglion neurons from all levels were isolated from C57BL/6J or *trpv4*^{-/-} mice¹ and cultured as described previously². (**A**, **C**, **D**) Neurons were loaded with fura2-AM (5 μM, 1 h) and imaged 24-48 h post-isolation; neurons were treated with HC 033031 (10 μM, >30 min, Tocris) to block TRPA1-dependent effects of 5'6'-EET³ and neurons were sequentially stimulated with 5'6'-EET or vehicle control (acetonitrile), followed by TRP channel agonists (TRPV4: GSK1016790A 100 nM; TRPV1: capsaicin 100 nM) and 70 mM KCl or treated with 5,6-EET, HC067047 or their vehicle as described in the manuscript (**B**). Proportion of neurons that responded to 5,6-EET (10 μM) by increasing calcium influx in wild-type or *trpv4*^{-/-} mice (**A**) or in sensory neurons pretreated with TRPV4 antagonist or its vehicle (**B**). Ca²⁺ responses of mice DRG neurons from wild type (**C**) or *trpv4*^{-/-} mice (**D**) to sequential stimulation. Data are represented as mean ± SEM. * significantly different from vehicle, p<0.05 (*), p<0.01 (**).

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