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***INPP4B* overexpression and *c-KIT* downregulation in human achalasia**

Running title: INPP4B and c-KIT dysregulation in achalasia

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21
22 39 **Abbreviations**

23
24 40 CPM - count per million, GO - gene ontology, LES - lower esophageal sphincter,
25
26 41 MEV - multiple experiment viewer, ICC - interstitial cells of Cajal, INPP4B - inositol
27
28 42 polyphosphate-4-phosphatase, type II B.
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33 44 **Correspondence address:**

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52 53 **Conflict of interest**

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54 54 The authors declare no conflict of interest
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3 **55 Author contribution**

4
5 56 EB, RDeG conceived the study, performed data analysis and wrote the manuscript;
6
7 57 AA, FB, VI, performed RNAseq and data analysis; AS, VB, FG, KVB, CE, EB
8
9 58 performed immunostaining and western blotting analysis; ML, AI, FT, GZ; AM,
10
11 59 MW, GEB, SM provided the clinical cases and tissue biopsies; SM, EB, RDeG, GEB,
12
13 60 MS, VS performed critical revision of the manuscript. All authors read the final
14
15 61 version of the paper.
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19

20 **63 Key points**

- 21
22 64 • Primary achalasia is a disorder due to neuronal defects supplying the
23
24 65 esophagus leading to altered peristalsis and lack of sphincter relaxation.
25
26 66 Nonetheless, the molecular mechanisms involved in this condition are poorly
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28 67 understood.
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31 68
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33 69 • Transcriptomic analysis of achalasic tissues identified a dysregulated
34
35 70 expression of different genes, in particular c-KIT (downregulated) and
36
37 71 INPP4B (upregulated), the latter being linked to Akt pathway regulation.
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42 73 • Our results unravel novel signaling pathways involved in the neuronal and
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44 74 interstitial cells of Cajal abnormalities in primary achalasia.
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3 80 **Abstract**

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5 81 **Background & Aims:** Achalasia is a rare motility disorder characterized by myenteric
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7 82 neuron and interstitial cells of Cajal (ICC) abnormalities leading to deranged/absent
8
9 83 peristalsis and lack of relaxation of the lower esophageal sphincter. The mechanisms
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11 84 contributing to neuronal and ICC changes in achalasia are only partially understood.
12
13 85 Our goal was to identify novel molecular features occurring in patients with primary
14
15 86 achalasia.

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18 87 **Methods:** Esophageal full-thickness biopsies from 42 (22 females; age range: 16-82
19
20 88 yrs) clinically, radiologically and manometrically characterized patients with primary
21
22 89 achalasia were examined and compared to those obtained from ten subjects (controls)
23
24 90 undergoing surgery for uncomplicated esophageal cancer (or upper stomach
25
26 91 disorders). Tissue RNA extracted from biopsies of cases and controls was used for
27
28 92 library preparation and sequencing. Data analysis was performed with the 'edgeR'
29
30 93 option of R-Bioconductor. Data were validated by real-time RT-PCR, western
31
32 94 blotting and immunohistochemistry.

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34
35 95 **Key Results:** Quantitative transcriptome evaluation and cluster analysis revealed 111
36
37 96 differentially expressed genes, with a $P \leq 10^{-3}$. Nine genes with a $P \leq 10^{-4}$ were further
38
39 97 validated. *CYR61*, *CTGF*, *c-KIT*, *DUSP5*, *EGR1* were downregulated, whereas
40
41 98 *AKAP6* and *INPP4B* were upregulated in patients vs controls. Compared to controls,
42
43 99 immunohistochemical analysis revealed a clear increase in INPP4B, whereas c-KIT
44
45 100 immunolabeling resulted downregulated. Since INPP4B regulates Akt pathway, we
46
47 101 used western blot to show that phospho-Akt was significantly reduced in achalasia
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49 102 patients vs controls.
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3 103 **Conclusions & Inferences:** The identification of altered gene expression, including
4
5 104 *INPP4B*, a regulator of the Akt pathway, highlights novel signaling pathways
6
7 105 involved in the neuronal and ICC changes underlying primary achalasia.
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9 106

11 107 **Key words:** Achalasia, cell signaling, c-KIT, INPP4B, transcriptome.
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For Peer Review

109 **Introduction**

110 Achalasia is a primary motility disorder of the esophagus occurring at any age, with
111 an estimated annual incidence of approximately 0.03-1 per 100 people and affecting
112 both sexes equally.^{1,2} Achalasia can be classified into primary forms (mostly
113 idiopathic), and cases associated with systemic disorders.^{3,4} From a pathogenetic
114 stand-point, achalasia is characterized by a predominant loss of inhibitory myenteric
115 neurons with a relative increase of cholinergic motoneurons⁵. Progressively
116 degenerative processes may involve virtually all neurons over time. These neuronal
117 changes result in an increase of the lower esophageal sphincter (LES) tone, which, in
118 conjunction with an altered peristalsis of the esophageal body, represent the
119 manometric correlate of any form of achalasia.⁶ Because of these functional
120 abnormalities patients with achalasia complain of dysphagia, regurgitation and chest
121 pain, often severe enough to require either surgery or pneumatic dilatation⁷.
122 Mechanisms leading to esophageal myenteric neurodegeneration and loss include
123 autoimmune responses likely triggered by environmental factors (e.g., neurotropic
124 viruses) in genetically predisposed individuals.^{2,3,8} In addition to neurons,
125 abnormalities of interstitial cells of Cajal (ICC), the pace-maker cells of the
126 gastrointestinal tract, were demonstrated in patients with achalasia⁹.

127
128 The current standard for diagnosing achalasia is high-resolution manometry, although
129 molecular biomarkers would be useful not only for diagnostic purposes, but also to
130 address targeted therapeutic options so far not yet available. Proteomic analysis of
131 sera from patients with achalasia and healthy individuals showed disease-related
132 upregulation of transthyretin (TTR), a carrier of thyroid hormone thyroxine and a
133 retinol-binding protein, associated with familial amyloid polyneuropathy.¹⁰ The

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3 134 observed upregulation may correlate with the consequent neural degeneration
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5 135 observed in achalasic patients.¹¹ Other studies demonstrated increased deposits of the
6
7 136 complement complex C5b-C9 and IgM within or proximal to ganglion cells of the
8
9 137 esophageal myenteric plexus.¹²
10
11 138 Achalasia due to rare genetic abnormalities includes recessive forms with mutations
12
13 139 in the neuronal nitric oxide synthase gene, *NOS1*¹³, or in *ALADIN* gene¹⁴ (Allgrove
14
15 140 syndrome or triple A syndrome, characterized by alacrima, achalasia and adrenal
16
17 141 cortex failure; OMIM #231550). Genome-wide and candidate gene association
18
19 142 studies have implicated the human leukocyte antigen (HLA) class II system as the
20
21 143 underlying genetic predisposing factor in achalasia. In particular, an 8-residues
22
23 144 insertion in the cytoplasmic tail of HLA-DQB1 has been identified as a strong risk
24
25 145 factor for achalasia, with a specific geospatial north-south gradient among
26
27 146 Europeans.¹⁵ Two amino acid substitutions in the extracellular domain of HLA-
28
29 147 DQ α 1, (lysine 41 encoded by HLA-DQA1*01:03) and of HLA-DQB1, (glutamic acid
30
31 148 45, encoded by HLA-DQB1*03:01 and HLA-DQB1*03:04) were characterized as
32
33 149 independent risk factors for achalasia and patients with the DQA1*0103 and
34
35 150 DQB1*0603 alleles have a significantly higher prevalence of anti-myenteric
36
37 151 antibodies.¹⁵⁻²⁰
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41 152 Nevertheless, the mechanisms contributing to the observed neuronal and ICC changes
42
43 153 in achalasia are still partially understood. The aim of this study was to identify the
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45 154 dysregulated molecular pathways / events occurring in primary (idiopathic) achalasia
46
47 155 to provide a molecular signature of primary achalasia.
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53 157 **Materials and Methods**
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55 158 **Patients and tissue biopsies**
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3 159 A number of 42 (n= 20 from Italy; n= 22 from Belgium; 22 females; age range: 16-82
4
5 160 yrs) patients with a well-established clinical, radiological and manometric diagnosis
6
7 161 of primary achalasia were recruited (Supplementary Table 1A). The included patients
8
9 162 were all of Caucasian origin. Each patient underwent surgery and 1-2 tissue samples
10
11 163 were obtained via a laparoscopic extramucosal Heller's myotomy extended 4-5 cm
12
13 164 along the distal esophagus (i.e., 1-2 cm full-thickness biopsy containing the circular
14
15 165 and longitudinal muscle with the associated enteric nervous system). Comparable
16
17 166 control tissues were obtained from the corresponding esophageal region of 10 (3
18
19 167 females; age range: 45-87 yrs) controls, who were referred to surgery for
20
21 168 uncomplicated esophageal cancer and upper stomach disorders, as reported in
22
23 169 **Supplementary Table 1B. Margins of all of sampled tissues were free of cancer.**
24
25
26 170 Signed informed consent was obtained for each subject enrolled in the study and data
27
28 171 handling was performed according to the Helsinki declaration. The study was
29
30 172 approved by the local Ethic Committee (study 42/2011/0/Oss protocol #877/211).

33 **RNA extraction**

34
35 174 Total RNA was extracted with the RiboPure kit from fresh frozen biopsies stored at -
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37 175 80°C in RNAlater, according to the manufacturer's instruction (Ambion,
38
39 176 ThermoFisher Scientific, Waltham, MA, USA). RNA was resuspended in 40 µl of
40
41 177 RNase-free water, and quality and quantity of RNA were evaluated on Agilent
42
43 178 Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA, USA). 150 ng of RNA was
44
45 179 used for each library preparation, according to the TruSeq RNA Sample Prep v2
46
47 180 protocol (Illumina, San Diego, CA, USA). Poly(A)-RNA molecules from 500 ng of
48
49 181 total RNA were purified using oligo-dT magnetic beads. Following purification, the
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51 182 mRNA was fragmented and randomly primed for reverse transcription followed by
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53 183 second-strand synthesis to create double-stranded cDNA fragments, that were then
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3 184 end-repaired and ligated using paired-end sequencing adapters. The products were
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5 185 then amplified to enrich for fragments carrying adapters ligated on both ends, thus
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7 186 creating the final cDNA library. RNAseq was performed on the HiScan SQ platform
8
9 187 (Illumina) as 75 bp paired-end.

11 188 **RNAseq data analysis**

13 189 Raw paired-end reads obtained by the HiScan SQ were filtered and trimmed with the
14
15 190 tool AdapterRemoval (<http://code.google.com/p/adaptremoval/>) to exclude low
16
17 191 quality bases and sequence adapters. Short reads were mapped on human reference
18
19 192 genome hg19 (<https://genome.ucsc.edu/>) with the pipeline Bowtie/Tophat
20
21 193 (<http://ccb.jhu.edu/software/tophat/index.shtml>) and Samtools
22
23 194 (<http://samtools.sourceforge.net/>) was adopted to remove optical/PCR duplicates.

24
25 195 Gene expression quantification was performed with the Python package htseq-count
26
27 196 (<http://www-huber.embl.de/HTSeq/doc/overview.html>) using the Ensembl release 72
28
29 197 annotation features (<http://www.ensembl.org>). The raw counts were normalized with
30
31 198 the R-Bioconductor package edgeR (function calcNormFactors) as “count per million”
32
33 199 (cpm). A filter procedure was adopted to analyze only genes with cpm > 3 in at least
34
35 200 2/4 samples. This process allowed to include 9616 genes, that were analyzed with the
36
37 201 R-Bioconductor package limma to determine the differentially expressed genes. In
38
39 202 particular, the function lmFit was used to fit the linear model, and the function eBayes
40
41 203 was adopted to compute the moderate t-statistic. Genes were ranked in order of
42
43 204 evidence for differential expression using log₂ fold change (log₂FC) and *P* value.²¹
44
45 205 Multiple Experiment Viewer (MEV) (v4.8.1) was used to perform the analysis in two
46
47 206 different ways: unsupervised hierarchical clustering (distance: Manhattan; clustering
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49 207 method: average) and principal component analysis. A total of 111 differentially
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51 208 expressed genes were selected ($P \leq 10^{-3}$) for the evaluation of the functional
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3 209 classification of biological processes and pathway over-representation, performed
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5 210 with the analytical tools PANTHER13.1 (Protein ANalysis THrough Evolutionary
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7 211 Relationships; <http://pantherdb.org>) and STRING. PANTHER is tightly integrated
8
9 212 with a number of different genomic resources: the InterPro Consortium of protein
10
11 213 classification resources, the Quest for Orthologs Consortium and the UniProt
12
13 214 Reference Proteome data sets. PANTHER Over-representation tool includes
14
15 215 functional annotations directly downloaded from the Gene Ontology (GO)
16
17 216 Consortium, in addition to the phylogenetically inferred annotations.²² Analysis was
18
19 217 performed using the option PANTHER Functional Classification and Over-
20
21 218 representation Tests, using the PANTHER GO-Slim Biological process and the
22
23 219 Fisher's Exact with false discovery rate (FDR) multiple test correction for statistical
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25 220 analysis. Independent analysis to observe protein-protein interaction was performed
26
27 221 with STRING, a database of known and predicted protein-protein interactions. The
28
29 222 interactions include direct (physical) and indirect (functional) associations; stemming
30
31 223 from computational prediction, from knowledge transfer between organisms, and
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33 224 from interactions aggregated from other (primary) databases.^{23,24}

225 **Quantitative real-time RT-qPCR**

226 *DNase I*-treated RNA (200 ng) was used for reverse transcription (RT) with random
227 hexamers using the Multiscribe RT system (ThermoFisher Scientific) at 48°C for 40
228 min in a final volume of 50 µl. *DNase I*-treated total RNA was obtained from a pool
229 of commercially available fresh frozen tissue biopsies of five controls subjects (single
230 vial purchased from AMSBIO, Abingdon, Oxford, UK; reference code: R1234106-P,
231 lot number B209050, human normal adult esophageal total RNA); the other RNAs
232 were obtained from fresh frozen tissue biopsies as described above.

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3 233 RT-qPCR was performed with SYBRGreen, 0.5 μ M primers, in an ABI 7500 Real-
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5 234 Time PCR System (ThermoFisher Scientific). All target genes were normalized with
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7 235 the endogenous control (beta-actin) using the $\Delta\Delta$ Ct comparative method. Primers are
8
9 236 available from the Authors on request.

237 **Western blotting**

238 Proteins were extracted from fresh frozen tissue biopsies of esophagus of achalasia
239 patients and controls by sonication using the Diagenode Bioruptor Pico sonicator
240 System (V 1.1) (10 cycles of 30 on/30 off; 20-60 kHz), in a volume of 50 μ l of Laemli
241 buffer 1X. A pool of normal esophageal tissues was commercially available (Abcam,
242 Cambridge, UK, reference code ab30243 - Human esophagus normal tissue lysate -
243 total protein), the other tissues analyzed with western blotting were obtained from
244 fresh frozen tissue biopsies of single cases and controls as reported in Supplementary
245 Tables 1A and 1B. Proteins were visualized on Coomassie Blue gel for quality and
246 quantity control. Western blot analysis was performed by SDS gel electrophoresis and
247 transfer onto nitrocellulose membrane, using the TransBlot system (Bio-Rad
248 Laboratories, Segrate, Italy). Primary antibodies used were the following: rabbit anti-
249 INPP4B diluted 1:200 (Abcam); rabbit anti-c-KIT (Abcam) diluted 1:200; mouse
250 anti-vinculin (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:5000; mouse anti-
251 GAPDH 1:10000 (Abcam); anti p-Akt (Ser473), anti p-Akt (Thr308), anti Akt (Cell
252 Signaling, Leiden, Netherlands), anti β -actin 1:10000 (Sigma-Aldrich). HRPO-
253 conjugated secondary antibodies (Sigma-Aldrich) were diluted 1:35000 in blocking
254 solution. Bands were visualized by the ECL method Supernova (Cyanagen, Bologna,
255 Italy) using the Chemidoc XRS+ Imager and densitometric analysis was performed
256 with Image lab Software (Bio-Rad Laboratories). All western blot analyses were

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3 257 performed at least in duplicate for each protein extract from each patient and control
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5 258 tissue biopsy.

6 7 259 **Immunohistochemistry**

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9 260 Immunohistochemistry was performed on paraffin-embedded adult human esophagus
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11 261 tissues according to protocols validated in our laboratory.²⁵ **Immunohistochemistry**
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13 262 **analyses and image acquisitions were performed in our laboratory at the University of**
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15 263 **Bologna.** Briefly, tissue sections were treated to remove paraffin embedding by
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17 264 sequential washes in xylene and graded ethanol. Antigen unmasking was performed
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19 265 by heating sections at 95°C in 10 mM Sodium citrate buffer, pH 6.0, for 10 min and
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21 266 subsequent cooling at room temperature for 30 min. To reduce endogenous
22
23 267 peroxidases, tissue sections were treated with an ad hoc blocking kit (GeneTex Inc.,
24
25 268 Irvine, CA, USA) and incubated for 16 h at 4°C with the same primary antibodies
26
27 269 used for western blotting, but diluted 1:50. 3,3'-diaminobenzidine staining was
28
29 270 performed according to standard protocols (DAB500 IHC Select® kit, Merck
30
31 271 Millipore, Darmstadt, Germany). Scores were assigned based on the intensity of DAB
32
33 272 staining (0 = no staining; + = 10-50% staining; ++ = 50-90% staining; +++ = ≥90%
34
35 273 staining of the areas; **all images were acquired at 10x magnification) according to**
36
37 274 **standard pathological assessment.**²⁶ **Three expert observers independently evaluated**
38
39 275 **the images (E.B., F.B. and R. De G.), and their analyses were blinded regarding**
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41 276 **control vs. achalasia state.** Image capturing of achalasia and control tissues was
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43 277 performed on a Nikon microscope using DS-5M digital camera (Nikon Instruments,
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45 278 Düsseldorf, Germany).

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51 52 280 **Statistical analysis**

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3 281 Quantitative difference in densitometry of western blots was performed with the
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5 282 Student's t-test option of GraphPad Quickstat calculator; statistical differences for the
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7 283 scores assigned to IHC samples from tissue biopsies was performed with Fisher's
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9 284 Test (4 x 2 contingency table) using the program Vassarstats.

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12 13 286 **Results**

14 15 287 *Whole transcriptome analysis via RNAseq*

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17 288 Total RNA extracted from esophageal biopsies of four cases (2F; age range: 30-58
18
19 289 yrs) and four controls (3F; age range: 27-50 yrs) was used for library preparation and
20
21 290 sequencing on Illumina HiScan SQ platform. Raw data showing the total amount of
22
23 291 reads/library and the mean coverage are reported in Supplementary Table 2, along
24
25 292 with the mean coverage for each sample. Quantitative data analysis using
26
27 293 Bioconductor from R package identified 111 genes with differential expression
28
29 294 between cases and controls at a $P \leq 10^{-3}$ (Table 1). Compared to controls, 77 genes
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31 295 were downregulated and 34 genes were upregulated in the achalasia group.
32
33 296 Hierarchical clustering considering the 111 genes was performed using MEV v4.8.1.
34
35 297 As shown in the heat map in Figure 1A, a concordant signature of up- and down-
36
37 298 regulation expression was evident between cases and controls. **Analysis using**
38
39 299 **PANTHER Functional classification test (PANTHER GO Slim Process) led us to**
40
41 300 **functionally map a total of 109 out of the 111 differentially expressed genes to**
42
43 301 **different biological processes, including, amongst others, cell adhesion, biogenesis,**
44
45 302 **developmental, immune and metabolic processes and response to stimuli**
46
47 303 **(<http://www.pantherdb.org/chart/pantherChart.jsp?listType=1&filterLevel=1&type=2>**
48
49 304 **&chartType=1&save=yes&basketItems=all&zoom=1.25&trackingId=4DCA8C9D00**
50
51 305 **F5C4649E3257CE82CFAC3E) (Figure 1B). PANTHER Over-representation Test**
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3 306 analysis was performed against the *H. sapiens* gene database, showing a set of over-
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5 307 represented biological processes in achalasia, including, amongst others,
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7 308 phosphorylation, the MAPK cascade signaling (including intracellular signal
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9 309 transduction and cell communication), regulation of phosphate metabolism and
10
11 310 developmental processes, e.g. cell differentiation and death (FDR < 0.05; Table 2).

12
13 311 Network analysis was performed with STRING using the list of genes (the previously
14
15 312 identified 111 genes) reported in Table 1, using the *H. sapiens* database as model
16
17 313 organism, and the “whole genome” option as statistical background. A number of 104
18
19 314 out of 111 genes matched the STRING protein database and were included in the
20
21 315 analysis (<https://string-db.org/cgi/network.pl?taskId=u9O5yLo4Lf2G>). Several
22
23 316 pathways were identified that showed a functional enrichment for biological
24
25 317 processes, such as regulation of development, cell differentiation, angiogenesis and
26
27 318 vascular architecture, with significant *P* values reported in Table 3. Many protein-
28
29 319 protein interactions converged on c-KIT (Figure 1C).

30
31 320 Among the dysregulated genes in achalasia, those differentially expressed with a
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33 321 significant $P \leq 10^{-4}$ were selected for further investigation, including *CYR61*, *CTGF*,
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35 322 *c-KIT*, *EGRI*, *LIF* and *CD69* (downregulated); and *AKAP6*, and *INPP4B*
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37 323 (overexpressed) (Table 1). To validate the data from whole transcriptome analysis, we
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39 324 performed quantitative real-time RT-qPCR using the total RNA of already sequenced
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41 325 samples (including two patients and one control). For each real-time RT-qPCR, raw
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43 326 data were normalized on a commercial pool of human normal esophagus RNA from
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45 327 fresh frozen tissue biopsies of five individuals (AMSBIO), using the $\Delta\Delta C_t$ method.
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47 328 Data reported in Figure 1D show independent technical replicates (same case-control
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49 329 samples, independent retro-transcription and real-time processes). A significant
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51 330 dysregulation of expression was confirmed for *c-KIT* and *INPP4B* (Figure 1D). These
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3 331 findings prompted us to focus on these two molecular targets, i.e. *c-KIT* and *INPP4B*,
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5 332 which were down- and up-regulated, respectively.
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9 334 ***Protein analysis of INPP4B and c-KIT confirmed the RNAseq data***

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11 335 The protein expression of c-KIT and INPP4B was studied by western blotting. In
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13 336 these experiments, control samples included a pool of normal esophageal tissues
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15 337 (Abcam) from five subjects in addition to control tissues obtained from single
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17 338 individual control subjects. Western blotting analysis for each tissue lysate was
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19 339 performed at least in two independent experiments, i.e. technical replicates. Equal
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21 340 amount of proteins was loaded for each sample and intensities were normalized using
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23 341 reference proteins. Representative examples of the protein patterns observed for c-
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25 342 KIT, INPP4B and reference proteins were reported in Figure 2A. Densitometric
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27 343 analysis of western blots was performed as indicated in Figure 2B. All cases and
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29 344 controls included in the western blotting analysis were reported in Supplementary
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31 345 Tables 1A and 1B. c-KIT downregulation was confirmed in tissue biopsies of
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33 346 achalasia patients compared to controls ($P= 0.0003$, Student's t-test; Figure 2B),
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35 347 whereas INPP4B overexpression was significantly detectable in achalasia patients vs.
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37 348 controls ($P=0.0381$; Student's t-test, Figure 2B).
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41 349 Immunohistochemistry analysis was performed in order to confirm the western blot
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43 350 data and to determine the cellular localization of the proteins herein identified by
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45 351 western blot. The density of c-KIT immunoreactive cells (mainly with the typical
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47 352 shape of ICC) was lower in achalasia than in controls (Fisher's exact test, $P= 0.0005$,
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49 353 Figure 3A). Compared to controls, INPP4B immunoreactivity was more intense in
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51 354 achalasia patients. In INPP4B-positive cases, the immunoreactive signal was present
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53 355 in different cells including muscle cells and myenteric ganglia. INPP4B expression in
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3 356 the normal tissues was very low or virtually absent (Fisher's exact test, $P= 0.0023$;
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5 357 Figure 3B).

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9 359 **Phospho-Akt is down regulated in achalasia**

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11 360 As INPP4B is a negative regulator of Akt signaling, we investigated Akt
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13 361 phosphorylation status. Equal amount of proteins was loaded for each sample and
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15 362 intensities were normalized using reference proteins. Representative examples of the
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17 363 phosphorylation patterns observed for the two phosphorylation sites in Akt (Ser473,
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19 364 and Thr308) were reported in Figure 4A. Densitometric analysis, performed
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21 365 according to our previously published data,²⁷ revealed that tissue samples from
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23 366 achalasia patients presented a reduced Akt phosphorylation, in particular the
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25 367 phosphorylation at Ser473 was significantly decreased compared to control tissues
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27 368 ($P=0.0495$, Student's t-test; Figure 4B).

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30 31 370 **Discussion**

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34 371 Primary achalasia causes significant impairment of esophageal motility leading to
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36 372 symptom generation and overall reduced patients' quality of life. This esophageal
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38 373 dysmotility of the esophagus has a complex etio-pathogenesis. Recent findings
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40 374 indicate that both an inflammatory / immune-mediated insult, as exhibited by
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42 375 myenteric ganglionitis (likely driven by neurotropic, e.g. herpes, virus infection)²⁸ and
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44 376 circulating anti-neuronal antibodies, play a pathogenetic role. A genetic predisposition
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46 377 conferred by HLA genetic background²⁰ can contribute to neural abnormalities
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48 378 underlying achalasia. A predominant genetic component has been identified in well-
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50 379 defined conditions such as 'triple A' (also referred to Allgrove's syndrome), where
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52 380 mutations in the ALADIN gene¹⁴ were found in familial clusters. Other data derive

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3 381 from *NOS1*, which encodes for the neuronal nitric oxide synthase. A stop codon
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5 382 mutation of this gene has been reported in a family where two children manifested a
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7 383 very severe clinical phenotype dominated by achalasia.¹³ Nevertheless, in the majority
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9 384 of cases the factors causing achalasia remain to be elucidated. As a consequence, the
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11 385 available therapeutic approaches are far from targeting the actual mechanisms leading
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13 386 to the neural impairment recognized to be the *primum movens* of this severe
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15 387 esophageal disorder. New understanding of achalasia, involving a deeper knowledge
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17 388 of the attendant molecular mechanisms, should aim to a better patient management
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19 389 and stop the disease at early stages. Therefore, the present study attempted to decipher
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21 390 the altered molecular pathways in patients with achalasia. We strategically used
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23 391 esophageal tissues from achalasia patients and examined gene expression via whole
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25 392 transcriptome analysis in order to provide novel targets for a therapeutic approach.
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27 393 From the RNAseq data we detected 111 genes with an altered expression. **Functional**
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29 394 **classification and over-representation analysis of the biological processes, where**
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31 395 **these genes mapped, was performed with two different bioinformatic tools,**
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33 396 **PANTHER and STRING. Results showed a significant over-representation of**
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35 397 **phosphorylation processes, intracellular signaling, cell communication and**
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37 398 **development /differentiation processes, including cell death. These data suggest that**
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39 399 **an imbalance of cell growth/survival vs. cell death** may cause the defects observed in
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41 400 the achalasia tissues, not only in term of neuron loss, but also as an overall tissue
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43 401 degeneration. **Notably, in a recent study, Palmieri et al. performed transcriptome**
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45 402 **analysis on tissues of primary achalasia patients and identified a significant**
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47 403 **enrichment of neuro-muscular and neuro-immune processes. These data support the**
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49 404 **concept that achalasia results from degenerative alterations / loss of myenteric**
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3 405 neurons mainly evoked by a chronic inflammatory infiltrate targeting the myenteric
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5 406 plexus.²⁹

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7 407 Among the genes with significant dysregulation, we confirmed by real-time qPCR *c-*
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9 408 *KIT* downregulation and *INPP4B* upregulation, respectively. *c-KIT* significant
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11 409 downregulation, validated both via western blot and immunohistochemistry, confirms
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13 410 and expands previously reported data showing a loss of ICC network in tissues of
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15 411 patients with primary achalasia.⁹ Our data highlighting a significant decrease of c-KIT
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17 412 expression in human achalasia support the concept that stem cell factor, the major
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19 413 ligand to c-KIT, cannot mediate ICC maintenance and survival. Although we did not
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21 414 look into specific apoptotic mechanisms, it is likely that a programmed cell death can
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23 415 involve ICC in achalasia.³⁰ Since ICC play a role in esophageal motility, their
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25 416 depletion can contribute to the deranged mechanisms leading to altered motility in
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27 417 achalasia.³¹

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31 418 The second gene for which we were able to confirm an altered expression at the
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33 419 protein level is INPP4B, encoding for inositol polyphosphate-4-phosphatase type II B,
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35 420 a negative regulator of Akt.^{32,33} The expression of this phosphatase was upregulated in
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37 421 many cell types in tissues from achalasic patients, indicating a general increase in
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39 422 inhibitory signals for cell survival, a mechanism stimulated by Akt-dependent
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41 423 pathways.³² Thus, an INPP4B increased expression can be correlated to the
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43 424 enrichment in genes involved in cell death. Notably, in achalasia tissues we observed
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45 425 a decrease in the phosphorylation of the Ser473 in Akt, which is a key
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47 426 phosphorylation step for its activation and function. Therefore, it may be postulated
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49 427 that in achalasia the altered molecular signaling led to an overall impaired cell
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51 428 survival, which, in the long term, generates a permanent tissue damage. Thus, one can
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53 429 assume that also ICCs, in addition to neurons, can be affected. Since many studies
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3 430 have investigated the Akt / INPP4B pathway as a potential “druggable” route to block
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5 431 tumor proliferation,^{33,34} the development of *ad hoc* molecules may become relevant
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7 432 for patients with primary achalasia.
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11 434 In conclusion, our study highlights the altered expression of two distinct genes, i.e. c-
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13 435 KIT and INPP4B down- and up-regulation, in patients with primary achalasia. Our
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15 436 results may provide a basis for future research aimed at investigating novel
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17 437 therapeutic targets for patients with primary achalasia.
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19 438

20 439 **Acknowledgements**

21
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24 441 Gori, and V. Bertakis and Ms. G. Galiazzo for technical help.
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31 444 **References**

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529 metastasis suppression through regulation of PI3K-AKT signaling at endosomes.
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For Peer Review

532 **Table 1. Genes differentially expressed in achalasia.**

533 Genes overexpressed in achalasia are labeled in red. Green shadowed area indicates

534 the genes with a $P \leq 10^{-4}$.

ENSEMBL transcript ID	GENE name	log2 Fold Change	P value
ENST00000451137	CYR61	-2.99	9.71E-06
ENST00000367976	CTGF	-2.74	1.19E-05
ENST00000513000	INPP4B	1.63	1.42E-04
ENST00000288135	KIT	-1.48	4.17E-04
ENST00000249075	LIF	-2.40	4.23E-04
ENST00000239938	EGR1	-1.95	4.65E-04
ENST00000228434	CD69	-3.84	4.94E-04
ENST00000280979	AKAP6	1.63	7.28E-04
ENST00000296464	HSPA4L	1.07	8.99E-04
ENST00000319653	FMN2	2.03	1.11E-03
ENST00000517956	FBXO32	1.26	1.12E-03
ENST00000265634	NPTX2	-1.88	1.20E-03
ENST00000550683	ACVRL1	-1.14	1.25E-03
ENST00000378827	BMP2	-1.99	1.51E-03
ENST00000371852	CH25H	-3.06	1.56E-03
ENST00000284669	KLHL41	2.43	1.58E-03
ENST00000436924	BRE	2.70	1.60E-03
ENST00000561981	FRRS1L	1.51	1.62E-03
ENST00000307792	SEMA3E	1.07	1.66E-03
ENST00000330871	SOCS3	-2.47	2.07E-03
ENST00000331569	ZNF703	1.18	2.30E-03
ENST00000305988	ADRB2	-1.41	2.47E-03
ENST00000420022	ADM5	-1.80	2.58E-03
ENST00000297316	SOX17	-1.82	2.76E-03
ENST00000252590	PLVAP	-1.29	2.77E-03
ENST00000256103	PMP2	-2.28	2.87E-03
ENST00000329099	FAM101B	-1.32	3.11E-03
ENST00000287814	TIMP4	-1.25	3.12E-03
ENST00000300177	GREM1	-2.58	3.29E-03
ENST00000485685	GGNBP2	-1.78	3.40E-03
ENST00000365328	RN7SK	-1.61	3.41E-03
ENST00000284878	CXADR	2.20	3.47E-03
ENST00000296046	CPA3	-1.76	3.52E-03
ENST00000302754	JUNB	-1.73	3.74E-03
ENST00000437551	PAX8-AS1	1.36	3.74E-03
ENST00000322507	COL12A1	-1.10	3.80E-03
ENST00000307637	C3AR1	-1.86	3.82E-03
ENST00000340695	SCXA	2.40	3.88E-03
ENST00000322954	UACA	0.94	3.89E-03

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ENST00000055682	KIAA2022	1.00	3.92E-03
ENST00000379374	PHEX	-3.10	3.98E-03
ENST00000357949	SERTAD1	-1.45	4.02E-03
ENST00000230990	HBEGF	-2.36	4.08E-03
ENST00000359534	KCNK5	-2.25	4.16E-03
ENST00000224605	GDF10	-1.64	4.17E-03
ENST00000396184	PDE1C	1.02	4.32E-03
ENST00000373509	PIM1	-2.29	4.33E-03
ENST00000408965	CEBPD	-1.45	4.59E-03
ENST00000267984	MESDC1	-1.17	4.82E-03
ENST00000329608	CSF1	-1.16	4.92E-03
ENST00000344120	SPRY4	-1.18	5.13E-03
ENST00000395076	PPM1A	0.85	5.16E-03
ENST00000350896	MSR1	-1.37	5.21E-03
ENST00000366667	AGT	-1.68	5.22E-03
ENST00000263370	ITPKC	-1.75	5.24E-03
ENST00000259989	FGFBP2	-1.75	5.24E-03
ENST00000311922	TRIB1	-2.74	5.24E-03
ENST00000608521	MIR663A	2.80	5.25E-03
ENST00000377103	THBD	-2.31	5.27E-03
ENST00000422542	AC005682,5	-1.34	5.32E-03
ENST00000396037	ST8SIA1	1.75	5.39E-03
ENST00000264805	PDE5A	2.61	5.58E-03
ENST00000400546	NCAM2	-1.27	5.65E-03
ENST00000369026	MCL1	-1.16	6.00E-03
ENST00000515896	RNA5-8SP6	1.12	6.20E-03
ENST00000399799	ROCK1	0.96	6.23E-03
ENST00000273153	CSRNP1	-1.74	6.25E-03
ENST00000244221	PAIP2B	1.09	6.25E-03
ENST00000376414	GPR183	-1.77	6.40E-03
ENST00000500741	DYNLL1-AS1	1.16	6.57E-03
ENST00000308086	THAP2	0.91	6.67E-03
ENST00000274063	SFRP2	-1.92	6.68E-03
ENST00000290551	BTG2	-1.53	6.72E-03
ENST00000380392	C2CD4B	-2.57	6.78E-03
ENST00000410087	CERKL	-1.36	6.83E-03
ENST00000367558	RGS16	-2.28	7.09E-03
ENST00000256257	RNF122	-1.65	7.10E-03
ENST00000367511	FAM129A	0.64	7.12E-03
ENST00000592790	VMP1	-2.93	7.17E-03
ENST00000323040	GPR4	-1.97	7.27E-03
ENST00000307851	HAVCR2	-1.68	7.40E-03
ENST00000324559	ANO5	0.96	7.62E-03
ENST00000328041	SLC24A3	-1.35	7.63E-03
ENST00000311734	IL1RL1	-3.50	7.72E-03
ENST00000373313	MAFB	-1.65	8.05E-03

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ENST00000382723	MSX1	-2.11	8.32E-03
ENST00000379731	B4GALT1	-1.49	8.33E-03
ENST00000329399	PDLIM1	-1.46	8.42E-03
ENST00000369583	DUSP5	-1.58	8.43E-03
ENST00000332029	SOCS1	-1.69	8.47E-03
ENST00000441535	FMO2	-1.27	8.55E-03
ENST00000256951	EMP1	-2.33	8.71E-03
ENST00000239223	DUSP1	-1.08	8.75E-03
ENST00000330106	CEND1	-2.85	8.84E-03
ENST00000498165	PPM1L	1.08	8.87E-03
ENST00000506002	MTND6P4	1.03	8.91E-03
ENST00000244050	SNAI1	-1.59	8.93E-03
ENST00000380874	FOXC1	-1.77	8.94E-03
ENST00000396073	ENAM	2.83	9.03E-03
ENST00000272928	ACKR3	-1.31	9.04E-03
ENST00000379359	RGCC	-1.11	9.22E-03
ENST00000333926	CISD1	0.80	9.30E-03
ENST00000367996	ADAMTS4	-4.18	9.37E-03
ENST00000380079	STEAP4	-2.07	9.38E-03
ENST00000413366	PRKCA	1.03	9.39E-03
ENST00000215794	USP18	-1.45	9.57E-03
ENST00000305352	S1PR1	-1.27	9.74E-03
ENST00000306065	ANKRD27	0.67	9.81E-03
ENST00000222390	HGF	-2.02	9.83E-03
ENST00000358432	EPHA2	-1.13	9.85E-03
ENST00000270001	ZFP14	0.88	9.89E-03

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536

537 **Table 2.** Output of PANTHER Over-representation test of biological processes
 538 enriched for the genes differentially expressed in achalasia tissues, compared to H.
 539 sapiens gene PANTHER database.
 540

PANTHER GO Slim Biological process	<i>H. sapiens</i>	Achalasia	Expected	Fold enrichment	Hierarchy (+/-)	raw P value	False Discovery Rate
Protein phosphorylation	81	4	.42	9.53	+	9.80e-04	3.99e-02
MAPK cascade	340	9	1.76	5.11	+	8.19e-05	6.66e-03
• intracellular signal transduction	1071	17	5.55	3.06	+	4.05e-05	4.94e-03
• signal transduction	2318	24	12.01	2.00	+	1.01e-03	3.54e-02
• Cell communication	2686	26	13.91	1.87	+	1.39e-03	3.76e-02
Regulation of catalytic activity	359	9	1.86	4.84	+	1.22e-04	7.47e-03
• regulation of molecular function	441	9	2.28	3.94	+	5.38e-04	2.62e-02
Regulation of phosphate metabolic process	537	12	2.78	4.31	+	2.64e-05	6.44e-03
Developmental process	1501	18	7.78	2.32	+	1.03e-03	3.13e-02

541
 542 Only results with False Discovery Rate<0.05 are reported in the table.
 543

544 **Table 3:** Output of STRING (protein-protein interaction and biological processes) for
 545 genes differentially regulated from whole transcriptome analysis of achalasia tissues.
 546

	Pathway ID and description	Count in gene set	False Discovery Rate P values
Biological Process (GO)	GO 0051094 positive regulation of developmental process	29	1.97e-10
	GO 1901342 regulation of vascular development	15	8.62e-10
	GO 0045597 positive regulation of cell differentiation	24	1.44e-09
	GO 004565 regulation of angiogenesis	14	2.7e-09
	GO 2000026 regulation of multicellular organismal development	31	3.31e-09
Molecular function (GO)	GO 0001228 transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	9	0.0483
	GO 0005515 protein binding	40	0.0483
	GO 0008083 growth factor activity	3	0.0483
Cellular component (GO)	GO 005615 extracellular space	20	0.00342
	GO 0009986 cell surface	14	0.00552

547
 548 Statistical background selected for the enrichment analysis set to “whole genome”.
 549

549 Figure Legends

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5 551 **Figure 1. Differentially expressed genes in achalasia tissue.** (A) Heat map showing
6
7 552 the concordant differential expression observed in the tissues from achalasia patients
8
9 553 compared to controls (for the genes reported in Table 1). Elaborated with MeV v4.8.1.
10
11 554 (B) Biological processes of genes (GO-Slim Biological processes) differentially
12
13 555 expressed in achalasia, as identified by PANTHER Functional Classification analysis.
14
15 556 (C) Graphical output of significantly enriched protein-protein interactions determined
16
17 557 with STRING analysis, showing the results of the 104 out of 111 dysregulated genes
18
19 558 in achalasia, compared with data present in the STRING databases. The different
20
21 559 colors in the figure inset identify the evidence for the different interactions (known;
22
23 560 predicted; identified with other means, such as co-expression, data mining, and
24
25 561 protein homology). The red square indicates c-KIT. (D) Real-time qRT-PCR data for
26
27 562 *c-KIT* and *INPP4B*. Data from case and control tissue biopsies were normalized on a
28
29 563 commercial pool of control tissue biopsies from esophagus. Student's t-test was
30
31 564 performed and *P* values are indicated. Bars indicate standard deviations.
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37 566 **Figure 2. Western blot analysis of differentially expressed genes.** (A) Example of
38
39 567 the protein expression profiles in achalasia tissues compared to control samples for c-
40
41 568 KIT and INPP4B. Western blotting was repeated in at least two independent
42
43 569 experiments. (B) Densitometric analysis of the relative intensities for c-KIT and
44
45 570 INPP4B. Quantitative data (mean values \pm standard deviation) from different
46
47 571 experiments obtained from each individual were compared using Student's t-test for
48
49 572 statistical analysis. For each sample, densitometric values were normalized on
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51 573 reference protein (vinculin). All experiments were carried out at least twice. Bars
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53 574 indicated standard deviations. *P* values are indicated in the graphs.
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5 576 **Figure 3. Immunostaining for c-KIT (A) and INPP4B (B).** (A) Examples of the
6
7 577 expression pattern for c-KIT in tissue biopsies from control (left) and achalasia
8
9 578 patients (right). Arrows indicate the positive staining for c-KIT. c-KIT staining color
10
11 579 histograms of controls (n=6) and cases (n=18) represent the number of samples
12
13 580 assessed according to the semi-quantitative score. c-KIT immunolabeling differences
14
15 581 in cases vs. controls were assessed by Fisher's exact test. (B) Examples of the
16
17 582 expression pattern for INPP4B in tissue biopsies from control (left) and achalasia
18
19 583 patients (right). INPP4B staining color histograms of controls (n=6) and cases (n=22)
20
21 584 represent the number of samples assessed according to the semi-quantitative score.
22
23 585 INPP4B immunolabeling differences in cases vs. controls were assessed by Fisher's
24
25 586 exact test. Scale bars are 100 μ m as indicated.
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33 588 **Figure 4. Phospho-Akt is down regulated in achalasia.** (A) Example of the protein
34
35 589 expression profiles in achalasia tissues compared to control samples of phospho-Akt
36
37 590 (Ser473; Thr308) and total Akt. (B) Quantitative data (mean values \pm standard
38
39 591 deviation) from different experiments (performed in duplicate) obtained from each
40
41 592 individual were compared using Student's t-test. For each sample, densitometric
42
43 593 values were normalized on reference protein (β -actin). The significantly different
44
45 594 relative phospho-Akt (Ser473) levels comparing achalasia vs. control tissues, were
46
47 595 reported in the histogram (P=0.0495, Student's t-test). Bars indicate standard
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49 596 deviations.
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***INPP4B* overexpression and *c-KIT* downregulation in human achalasia**

Running title: INPP4B and c-KIT dysregulation in achalasia

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22 39 **Abbreviations**

23
24 40 CPM - count per million, GO - gene ontology, LES - lower esophageal sphincter,
25
26 41 MEV - multiple experiment viewer, ICC - interstitial cells of Cajal, INPP4B - inositol
27
28 42 polyphosphate-4-phosphatase, type II B.
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52 53 **Conflict of interest**

53
54 54 The authors declare no conflict of interest
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3 **55 Author contribution**

4
5 56 EB, RDeG conceived the study, performed data analysis and wrote the manuscript;
6
7 57 AA, FB, VI, performed RNAseq and data analysis; AS, VB, FG, KVB, CE, EB
8
9 58 performed immunostaining and western blotting analysis; ML, AI, FT, GZ; AM,
10
11 59 MW, GEB, SM provided the clinical cases and tissue biopsies; SM, EB, RDeG, GEB,
12
13 60 MS, VS performed critical revision of the manuscript. All authors read the final
14
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16 61 version of the paper.
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20 **63 Key points**

- 21
22 64 • Primary achalasia is a disorder due to neuronal defects supplying the
23
24 65 esophagus leading to altered peristalsis and lack of sphincter relaxation.
25
26 66 Nonetheless, the molecular mechanisms involved in this condition are poorly
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28 67 understood.
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33 69 • Transcriptomic analysis of achalasic tissues identified a dysregulated
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35 70 expression of different genes, in particular c-KIT (downregulated) and
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37 71 INPP4B (upregulated), the latter being linked to Akt pathway regulation.
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42 73 • Our results unravel novel signaling pathways involved in the neuronal and
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44 74 interstitial cells of Cajal abnormalities in primary achalasia.
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3 80 **Abstract**

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5 81 **Background & Aims:** Achalasia is a rare motility disorder characterized by myenteric
6
7 82 neuron and interstitial cells of Cajal (ICC) abnormalities leading to deranged/absent
8
9 83 peristalsis and lack of relaxation of the lower esophageal sphincter. The mechanisms
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11 84 contributing to neuronal and ICC changes in achalasia are only partially understood.
12
13 85 Our goal was to identify novel molecular features occurring in patients with primary
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15 86 achalasia.

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18 87 **Methods:** Esophageal full-thickness biopsies from 42 (22 females; age range: 16-82
19
20 88 yrs) clinically, radiologically and manometrically characterized patients with primary
21
22 89 achalasia were examined and compared to those obtained from ten subjects (controls)
23
24 90 undergoing surgery for uncomplicated esophageal cancer (or upper stomach
25
26 91 disorders). Tissue RNA extracted from biopsies of cases and controls was used for
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28 92 library preparation and sequencing. Data analysis was performed with the 'edgeR'
29
30 93 option of R-Bioconductor. Data were validated by real-time RT-PCR, western
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32 94 blotting and immunohistochemistry.

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34
35 95 **Key Results:** Quantitative transcriptome evaluation and cluster analysis revealed 111
36
37 96 differentially expressed genes, with a $P \leq 10^{-3}$. Nine genes with a $P \leq 10^{-4}$ were further
38
39 97 validated. *CYR61*, *CTGF*, *c-KIT*, *DUSP5*, *EGR1* were downregulated, whereas
40
41 98 *AKAP6* and *INPP4B* were upregulated in patients vs controls. Compared to controls,
42
43 99 immunohistochemical analysis revealed a clear increase in INPP4B, whereas c-KIT
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45 100 immunolabeling resulted downregulated. Since INPP4B regulates Akt pathway, we
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47 101 used western blot to show that phospho-Akt was significantly reduced in achalasia
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49 102 patients vs controls.
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3 103 **Conclusions & Inferences:** The identification of altered gene expression, including
4
5 104 *INPP4B*, a regulator of the Akt pathway, highlights novel signaling pathways
6
7 105 involved in the neuronal and ICC changes underlying primary achalasia.
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11 107 **Key words:** Achalasia, cell signaling, c-KIT, INPP4B, transcriptome.
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For Peer Review

109 **Introduction**

110 Achalasia is a primary motility disorder of the esophagus occurring at any age, with
111 an estimated annual incidence of approximately 0.03-1 per 100 people and affecting
112 both sexes equally.^{1,2} Achalasia can be classified into primary forms (mostly
113 idiopathic), and cases associated with systemic disorders.^{3,4} From a pathogenetic
114 stand-point, achalasia is characterized by a predominant loss of inhibitory myenteric
115 neurons with a relative increase of cholinergic motoneurons⁵. Progressively
116 degenerative processes may involve virtually all neurons over time. These neuronal
117 changes result in an increase of the lower esophageal sphincter (LES) tone, which, in
118 conjunction with an altered peristalsis of the esophageal body, represent the
119 manometric correlate of any form of achalasia.⁶ Because of these functional
120 abnormalities patients with achalasia complain of dysphagia, regurgitation and chest
121 pain, often severe enough to require either surgery or pneumatic dilatation⁷.
122 Mechanisms leading to esophageal myenteric neurodegeneration and loss include
123 autoimmune responses likely triggered by environmental factors (e.g., neurotropic
124 viruses) in genetically predisposed individuals.^{2,3,8} In addition to neurons,
125 abnormalities of interstitial cells of Cajal (ICC), the pace-maker cells of the
126 gastrointestinal tract, were demonstrated in patients with achalasia⁹.

127
128 The current standard for diagnosing achalasia is high-resolution manometry, although
129 molecular biomarkers would be useful not only for diagnostic purposes, but also to
130 address targeted therapeutic options so far not yet available. Proteomic analysis of
131 sera from patients with achalasia and healthy individuals showed disease-related
132 upregulation of transthyretin (TTR), a carrier of thyroid hormone thyroxine and a
133 retinol-binding protein, associated with familial amyloid polyneuropathy.¹⁰ The

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2
3 134 observed upregulation may correlate with the consequent neural degeneration
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5 135 observed in achalasic patients.¹¹ Other studies demonstrated increased deposits of the
6
7 136 complement complex C5b-C9 and IgM within or proximal to ganglion cells of the
8
9 137 esophageal myenteric plexus.¹²
10
11 138 Achalasia due to rare genetic abnormalities includes recessive forms with mutations
12
13 139 in the neuronal nitric oxide synthase gene, *NOS1*¹³, or in *ALADIN* gene¹⁴ (Allgrove
14
15 140 syndrome or triple A syndrome, characterized by alacrima, achalasia and adrenal
16
17 141 cortex failure; OMIM #231550). Genome-wide and candidate gene association
18
19 142 studies have implicated the human leukocyte antigen (HLA) class II system as the
20
21 143 underlying genetic predisposing factor in achalasia. In particular, an 8-residues
22
23 144 insertion in the cytoplasmic tail of HLA-DQB1 has been identified as a strong risk
24
25 145 factor for achalasia, with a specific geospatial north-south gradient among
26
27 146 Europeans.¹⁵ Two amino acid substitutions in the extracellular domain of HLA-
28
29 147 DQ α 1, (lysine 41 encoded by HLA-DQA1*01:03) and of HLA-DQB1, (glutamic acid
30
31 148 45, encoded by HLA-DQB1*03:01 and HLA-DQB1*03:04) were characterized as
32
33 149 independent risk factors for achalasia and patients with the DQA1*0103 and
34
35 150 DQB1*0603 alleles have a significantly higher prevalence of anti-myenteric
36
37 151 antibodies.¹⁵⁻²⁰
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41 152 Nevertheless, the mechanisms contributing to the observed neuronal and ICC changes
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43 153 in achalasia are still partially understood. The aim of this study was to identify the
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45 154 dysregulated molecular pathways / events occurring in primary (idiopathic) achalasia
46
47 155 to provide a molecular signature of primary achalasia.
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53 157 **Materials and Methods**
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55 158 **Patients and tissue biopsies**
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3 159 A number of 42 (n= 20 from Italy; n= 22 from Belgium; 22 females; age range: 16-82
4
5 160 yrs) patients with a well-established clinical, radiological and manometric diagnosis
6
7 161 of primary achalasia were recruited (Supplementary Table 1A). The included patients
8
9 162 were all of Caucasian origin. Each patient underwent surgery and 1-2 tissue samples
10
11 163 were obtained via a laparoscopic extramucosal Heller's myotomy extended 4-5 cm
12
13 164 along the distal esophagus (i.e., 1-2 cm full-thickness biopsy containing the circular
14
15 165 and longitudinal muscle with the associated enteric nervous system). Comparable
16
17 166 control tissues were obtained from the corresponding esophageal region of 10 (3
18
19 167 females; age range: 45-87 yrs) controls, who were referred to surgery for
20
21 168 uncomplicated esophageal cancer and upper stomach disorders, as reported in
22
23 169 Supplementary Table 1B. Margins of all of sampled tissues were free of cancer.
24
25
26 170 Signed informed consent was obtained for each subject enrolled in the study and data
27
28 171 handling was performed according to the Helsinki declaration. The study was
29
30 172 approved by the local Ethic Committee (study 42/2011/0/Oss protocol #877/211).

33 173 **RNA extraction**

34
35 174 Total RNA was extracted with the RiboPure kit from fresh frozen biopsies stored at -
36
37 175 80°C in RNAlater, according to the manufacturer's instruction (Ambion,
38
39 176 ThermoFisher Scientific, Waltham, MA, USA). RNA was resuspended in 40 µl of
40
41 177 RNase-free water, and quality and quantity of RNA were evaluated on Agilent
42
43 178 Bioanalyzer 2100 instrument (Agilent, Santa Clara, CA, USA). 150 ng of RNA was
44
45 179 used for each library preparation, according to the TruSeq RNA Sample Prep v2
46
47 180 protocol (Illumina, San Diego, CA, USA). Poly(A)-RNA molecules from 500 ng of
48
49 181 total RNA were purified using oligo-dT magnetic beads. Following purification, the
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51 182 mRNA was fragmented and randomly primed for reverse transcription followed by
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53 183 second-strand synthesis to create double-stranded cDNA fragments, that were then
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3 184 end-repaired and ligated using paired-end sequencing adapters. The products were
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5 185 then amplified to enrich for fragments carrying adapters ligated on both ends, thus
6
7 186 creating the final cDNA library. RNAseq was performed on the HiScan SQ platform
8
9 187 (Illumina) as 75 bp paired-end.

11 188 **RNAseq data analysis**

13 189 Raw paired-end reads obtained by the HiScan SQ were filtered and trimmed with the
14
15 190 tool AdapterRemoval (<http://code.google.com/p/adaptremoval/>) to exclude low
16
17 191 quality bases and sequence adapters. Short reads were mapped on human reference
18
19 192 genome hg19 (<https://genome.ucsc.edu/>) with the pipeline Bowtie/Tophat
20
21 193 (<http://ccb.jhu.edu/software/tophat/index.shtml>) and Samtools
22
23 194 (<http://samtools.sourceforge.net/>) was adopted to remove optical/PCR duplicates.
24
25 195 Gene expression quantification was performed with the Python package htseq-count
26
27 196 (<http://www-huber.embl.de/HTSeq/doc/overview.html>) using the Ensembl release 72
28
29 197 annotation features (<http://www.ensembl.org>). The raw counts were normalized with
30
31 198 the R-Bioconductor package edgeR (function calcNormFactors) as “count per million”
32
33 199 (cpm). A filter procedure was adopted to analyze only genes with cpm > 3 in at least
34
35 200 2/4 samples. This process allowed to include 9616 genes, that were analyzed with the
36
37 201 R-Bioconductor package limma to determine the differentially expressed genes. In
38
39 202 particular, the function lmFit was used to fit the linear model, and the function eBayes
40
41 203 was adopted to compute the moderate t-statistic. Genes were ranked in order of
42
43 204 evidence for differential expression using log₂ fold change (log₂FC) and *P* value.²¹
44
45 205 Multiple Experiment Viewer (MEV) (v4.8.1) was used to perform the analysis in two
46
47 206 different ways: unsupervised hierarchical clustering (distance: Manhattan; clustering
48
49 207 method: average) and principal component analysis. A total of 111 differentially
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51 208 expressed genes were selected ($P \leq 10^{-3}$) for the evaluation of the functional
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3 209 classification of biological processes and pathway over-representation, performed
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5 210 with the analytical tools PANTHER13.1 (Protein ANalysis THrough Evolutionary
6
7 211 Relationships; <http://pantherdb.org>) and STRING. PANTHER is tightly integrated
8
9 212 with a number of different genomic resources: the InterPro Consortium of protein
10
11 213 classification resources, the Quest for Orthologs Consortium and the UniProt
12
13 214 Reference Proteome data sets. PANTHER Over-representation tool includes
14
15 215 functional annotations directly downloaded from the Gene Ontology (GO)
16
17 216 Consortium, in addition to the phylogenetically inferred annotations.²² Analysis was
18
19 217 performed using the option PANTHER Functional Classification and Over-
20
21 218 representation Tests, using the PANTHER GO-Slim Biological process and the
22
23 219 Fisher's Exact with false discovery rate (FDR) multiple test correction for statistical
24
25 220 analysis. Independent analysis to observe protein-protein interaction was performed
26
27 221 with STRING, a database of known and predicted protein-protein interactions. The
28
29 222 interactions include direct (physical) and indirect (functional) associations; stemming
30
31 223 from computational prediction, from knowledge transfer between organisms, and
32
33 224 from interactions aggregated from other (primary) databases.^{23,24}

225 **Quantitative real-time RT-qPCR**

226 *DNase I*-treated RNA (200 ng) was used for reverse transcription (RT) with random
227 hexamers using the Multiscribe RT system (ThermoFisher Scientific) at 48°C for 40
228 min in a final volume of 50 µl. *DNase I*-treated total RNA was obtained from a pool
229 of commercially available fresh frozen tissue biopsies of five controls subjects (single
230 vial purchased from AMSBIO, Abingdon, Oxford, UK; reference code: R1234106-P,
231 lot number B209050, human normal adult esophageal total RNA); the other RNAs
232 were obtained from fresh frozen tissue biopsies as described above.

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3 233 RT-qPCR was performed with SYBRGreen, 0.5 μ M primers, in an ABI 7500 Real-
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5 234 Time PCR System (ThermoFisher Scientific). All target genes were normalized with
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7 235 the endogenous control (beta-actin) using the $\Delta\Delta$ Ct comparative method. Primers are
8
9 236 available from the Authors on request.

11 237 **Western blotting**

12
13 238 Proteins were extracted from fresh frozen tissue biopsies of esophagus of achalasia
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15 239 patients and controls by sonication using the Diagenode Bioruptor Pico sonicator
16
17 240 System (V 1.1) (10 cycles of 30 on/30 off; 20-60 kHz), in a volume of 50 μ l of Laemli
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19 241 buffer 1X. A pool of normal esophageal tissues was commercially available (Abcam,
20
21 242 Cambridge, UK, reference code ab30243 - Human esophagus normal tissue lysate -
22
23 243 total protein), the other tissues analyzed with western blotting were obtained from
24
25 244 fresh frozen tissue biopsies of single cases and controls as reported in Supplementary
26
27 245 Tables 1A and 1B. Proteins were visualized on Coomassie Blue gel for quality and
28
29 246 quantity control. Western blot analysis was performed by SDS gel electrophoresis and
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31 247 transfer onto nitrocellulose membrane, using the TransBlot system (Bio-Rad
32
33 248 Laboratories, Segrate, Italy). Primary antibodies used were the following: rabbit anti-
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35 249 INPP4B diluted 1:200 (Abcam); rabbit anti-c-KIT (Abcam) diluted 1:200; mouse
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37 250 anti-vinculin (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:5000; mouse anti-
38
39 251 GAPDH 1:10000 (Abcam); anti p-Akt (Ser473), anti p-Akt (Thr308), anti Akt (Cell
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41 252 Signaling, Leiden, Netherlands), anti β -actin 1:10000 (Sigma-Aldrich). HRPO-
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43 253 conjugated secondary antibodies (Sigma-Aldrich) were diluted 1:35000 in blocking
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45 254 solution. Bands were visualized by the ECL method Supernova (Cyanagen, Bologna,
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47 255 Italy) using the Chemidoc XRS+ Imager and densitometric analysis was performed
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49 256 with Image lab Software (Bio-Rad Laboratories). All western blot analyses were
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3 257 performed at least in duplicate for each protein extract from each patient and control
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5 258 tissue biopsy.

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7 259 **Immunohistochemistry**

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9 260 Immunohistochemistry was performed on paraffin-embedded adult human esophagus
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11 261 tissues according to protocols validated in our laboratory.²⁵ Immunohistochemistry
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13 262 analyses and image acquisitions were performed in our laboratory at the University of
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15 263 Bologna. Briefly, tissue sections were treated to remove paraffin embedding by
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17 264 sequential washes in xylene and graded ethanol. Antigen unmasking was performed
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19 265 by heating sections at 95°C in 10 mM Sodium citrate buffer, pH 6.0, for 10 min and
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21 266 subsequent cooling at room temperature for 30 min. To reduce endogenous
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23 267 peroxidases, tissue sections were treated with an ad hoc blocking kit (GeneTex Inc.,
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25 268 Irvine, CA, USA) and incubated for 16 h at 4°C with the same primary antibodies
26
27 269 used for western blotting, but diluted 1:50. 3,3'-diaminobenzidine staining was
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29 270 performed according to standard protocols (DAB500 IHC Select® kit, Merck
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31 271 Millipore, Darmstadt, Germany). Scores were assigned based on the intensity of DAB
32
33 272 staining (0 = no staining; + = 10-50% staining; ++ = 50-90% staining; +++ = ≥90%
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35 273 staining of the areas; all images were acquired at 10x magnification) according to
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37 274 standard pathological assessment.²⁶ Three expert observers independently evaluated
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39 275 the images (E.B., F.B. and R. De G.), and their analyses were blinded regarding
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41 276 control vs. achalasia state. Image capturing of achalasia and control tissues was
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43 277 performed on a Nikon microscope using DS-5M digital camera (Nikon Instruments,
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45 278 Düsseldorf, Germany).

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52 280 **Statistical analysis**

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3 281 Quantitative difference in densitometry of western blots was performed with the
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5 282 Student's t-test option of GraphPad Quickstat calculator; statistical differences for the
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7 283 scores assigned to IHC samples from tissue biopsies was performed with Fisher's
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9 284 Test (4 x 2 contingency table) using the program Vassarstats.

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12 13 286 **Results**

14 15 287 *Whole transcriptome analysis via RNAseq*

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17 288 Total RNA extracted from esophageal biopsies of four cases (2F; age range: 30-58
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19 289 yrs) and four controls (3F; age range: 27-50 yrs) was used for library preparation and
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21 290 sequencing on Illumina HiScan SQ platform. Raw data showing the total amount of
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23 291 reads/library and the mean coverage are reported in Supplementary Table 2, along
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25 292 with the mean coverage for each sample. Quantitative data analysis using
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27 293 Bioconductor from R package identified 111 genes with differential expression
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29 294 between cases and controls at a $P \leq 10^{-3}$ (Table 1). Compared to controls, 77 genes
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31 295 were downregulated and 34 genes were upregulated in the achalasia group.
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33 296 Hierarchical clustering considering the 111 genes was performed using MEV v4.8.1.
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35 297 As shown in the heat map in Figure 1A, a concordant signature of up- and down-
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37 298 regulation expression was evident between cases and controls. Analysis using
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39 299 PANTHER Functional classification test (PANTHER GO Slim Process) led us to
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41 300 functionally map a total of 109 out of the 111 differentially expressed genes to
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43 301 different biological processes, including, amongst others, cell adhesion, biogenesis,
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45 302 developmental, immune and metabolic processes and response to stimuli
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47 303 (<http://www.pantherdb.org/chart/pantherChart.jsp?listType=1&filterLevel=1&type=2>
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49 304 &chartType=1&save=yes&basketItems=all&zoom=1.25&trackingId=4DCA8C9D00
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51 305 F5C4649E3257CE82CFAC3E) (Figure 1B). PANTHER Over-representation Test
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3 306 analysis was performed against the *H. sapiens* gene database, showing a set of over-
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5 307 represented biological processes in achalasia, including, amongst others,
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7 308 phosphorylation, the MAPK cascade signaling (including intracellular signal
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9 309 transduction and cell communication), regulation of phosphate metabolism and
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11 310 developmental processes, e.g. cell differentiation and death (FDR < 0.05; Table 2).

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13 311 Network analysis was performed with STRING using the list of genes (the previously
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15 312 identified 111 genes) reported in Table 1, using the *H. sapiens* database as model
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17 313 organism, and the “whole genome” option as statistical background. A number of 104
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19 314 out of 111 genes matched the STRING protein database and were included in the
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21 315 analysis (<https://string-db.org/cgi/network.pl?taskId=u9O5yLo4Lf2G>). Several
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23 316 pathways were identified that showed a functional enrichment for biological
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25 317 processes, such as regulation of development, cell differentiation, angiogenesis and
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27 318 vascular architecture, with significant *P* values reported in Table 3. Many protein-
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29 319 protein interactions converged on c-KIT (Figure 1C).

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31 320 Among the dysregulated genes in achalasia, those differentially expressed with a
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33 321 significant $P \leq 10^{-4}$ were selected for further investigation, including *CYR61*, *CTGF*,
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35 322 *c-KIT*, *EGRI*, *LIF* and *CD69* (downregulated); and *AKAP6*, and *INPP4B*
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37 323 (overexpressed) (Table 1). To validate the data from whole transcriptome analysis, we
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39 324 performed quantitative real-time RT-qPCR using the total RNA of already sequenced
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41 325 samples (including two patients and one control). For each real-time RT-qPCR, raw
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43 326 data were normalized on a commercial pool of human normal esophagus RNA from
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45 327 fresh frozen tissue biopsies of five individuals (AMSBIO), using the $\Delta\Delta C_t$ method.
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47 328 Data reported in Figure 1D show independent technical replicates (same case-control
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49 329 samples, independent retro-transcription and real-time processes). A significant
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51 330 dysregulation of expression was confirmed for *c-KIT* and *INPP4B* (Figure 1D). These
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3 331 findings prompted us to focus on these two molecular targets, i.e. *c-KIT* and *INPP4B*,
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5 332 which were down- and up-regulated, respectively.
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9 334 ***Protein analysis of INPP4B and c-KIT confirmed the RNAseq data***

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11 335 The protein expression of c-KIT and INPP4B was studied by western blotting. In
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13 336 these experiments, control samples included a pool of normal esophageal tissues
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15 337 (Abcam) from five subjects in addition to control tissues obtained from single
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17 338 individual control subjects. Western blotting analysis for each tissue lysate was
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19 339 performed at least in two independent experiments, i.e. technical replicates. Equal
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21 340 amount of proteins was loaded for each sample and intensities were normalized using
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23 341 reference proteins. Representative examples of the protein patterns observed for c-
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25 342 KIT, INPP4B and reference proteins were reported in Figure 2A. Densitometric
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27 343 analysis of western blots was performed as indicated in Figure 2B. All cases and
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29 344 controls included in the western blotting analysis were reported in Supplementary
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31 345 Tables 1A and 1B. c-KIT downregulation was confirmed in tissue biopsies of
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33 346 achalasia patients compared to controls ($P= 0.0003$, Student's t-test; Figure 2B),
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35 347 whereas INPP4B overexpression was significantly detectable in achalasia patients vs.
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37 348 controls ($P=0.0381$; Student's t-test, Figure 2B).
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41 349 Immunohistochemistry analysis was performed in order to confirm the western blot
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43 350 data and to determine the cellular localization of the proteins herein identified by
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45 351 western blot. The density of c-KIT immunoreactive cells (mainly with the typical
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47 352 shape of ICC) was lower in achalasia than in controls (Fisher's exact test, $P= 0.0005$,
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49 353 Figure 3A). Compared to controls, INPP4B immunoreactivity was more intense in
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51 354 achalasia patients. In INPP4B-positive cases, the immunoreactive signal was present
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53 355 in different cells including muscle cells and myenteric ganglia. INPP4B expression in
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3 356 the normal tissues was very low or virtually absent (Fisher's exact test, $P= 0.0023$;
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5 357 Figure 3B).

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9 359 **Phospho-Akt is down regulated in achalasia**

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11 360 As INPP4B is a negative regulator of Akt signaling, we investigated Akt
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13 361 phosphorylation status. Equal amount of proteins was loaded for each sample and
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15 362 intensities were normalized using reference proteins. Representative examples of the
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17 363 phosphorylation patterns observed for the two phosphorylation sites in Akt (Ser473,
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19 364 and Thr308) were reported in Figure 4A. Densitometric analysis, performed
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21 365 according to our previously published data,²⁷ revealed that tissue samples from
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23 366 achalasia patients presented a reduced Akt phosphorylation, in particular the
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25 367 phosphorylation at Ser473 was significantly decreased compared to control tissues
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27 368 ($P=0.0495$, Student's t-test; Figure 4B).

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32 33 370 **Discussion**

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35 371 Primary achalasia causes significant impairment of esophageal motility leading to
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37 372 symptom generation and overall reduced patients' quality of life. This esophageal
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39 373 dysmotility of the esophagus has a complex etio-pathogenesis. Recent findings
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41 374 indicate that both an inflammatory / immune-mediated insult, as exhibited by
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43 375 myenteric ganglionitis (likely driven by neurotropic, e.g. herpes, virus infection)²⁸ and
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45 376 circulating anti-neuronal antibodies, play a pathogenetic role. A genetic predisposition
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47 377 conferred by HLA genetic background²⁰ can contribute to neural abnormalities
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49 378 underlying achalasia. A predominant genetic component has been identified in well-
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51 379 defined conditions such as 'triple A' (also referred to Allgrove's syndrome), where
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53 380 mutations in the ALADIN gene¹⁴ were found in familial clusters. Other data derive
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3 381 from *NOS1*, which encodes for the neuronal nitric oxide synthase. A stop codon
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5 382 mutation of this gene has been reported in a family where two children manifested a
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7 383 very severe clinical phenotype dominated by achalasia.¹³ Nevertheless, in the majority
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9 384 of cases the factors causing achalasia remain to be elucidated. As a consequence, the
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11 385 available therapeutic approaches are far from targeting the actual mechanisms leading
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13 386 to the neural impairment recognized to be the *primum movens* of this severe
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15 387 esophageal disorder. New understanding of achalasia, involving a deeper knowledge
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17 388 of the attendant molecular mechanisms, should aim to a better patient management
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19 389 and stop the disease at early stages. Therefore, the present study attempted to decipher
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21 390 the altered molecular pathways in patients with achalasia. We strategically used
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23 391 esophageal tissues from achalasia patients and examined gene expression via whole
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25 392 transcriptome analysis in order to provide novel targets for a therapeutic approach.
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27 393 From the RNAseq data we detected 111 genes with an altered expression. Functional
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29 394 classification and over-representation analysis of the biological processes, where
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31 395 these genes mapped, was performed with two different bioinformatic tools,
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33 396 PANTHER and STRING. Results showed a significant over-representation of
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35 397 phosphorylation processes, intracellular signaling, cell communication and
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37 398 development /differentiation processes, including cell death. These data suggest that
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39 399 an imbalance of cell growth/survival vs. cell death may cause the defects observed in
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41 400 the achalasia tissues, not only in term of neuron loss, but also as an overall tissue
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43 401 degeneration. Notably, in a recent study, Palmieri et al. performed transcriptome
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45 402 analysis on tissues of primary achalasia patients and identified a significant
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47 403 enrichment of neuro-muscular and neuro-immune processes. These data support the
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49 404 concept that achalasia results from degenerative alterations / loss of myenteric
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3 405 neurons mainly evoked by a chronic inflammatory infiltrate targeting the myenteric
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5 406 plexus.²⁹

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7 407 Among the genes with significant dysregulation, we confirmed by real-time qPCR *c-*
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9 408 *KIT* downregulation and *INPP4B* upregulation, respectively. *c-KIT* significant
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11 409 downregulation, validated both via western blot and immunohistochemistry, confirms
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13 410 and expands previously reported data showing a loss of ICC network in tissues of
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15 411 patients with primary achalasia.⁹ Our data highlighting a significant decrease of c-KIT
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17 412 expression in human achalasia support the concept that stem cell factor, the major
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19 413 ligand to c-KIT, cannot mediate ICC maintenance and survival. Although we did not
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21 414 look into specific apoptotic mechanisms, it is likely that a programmed cell death can
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23 415 involve ICC in achalasia.³⁰ Since ICC play a role in esophageal motility, their
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25 416 depletion can contribute to the deranged mechanisms leading to altered motility in
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27 417 achalasia.³¹

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31 418 The second gene for which we were able to confirm an altered expression at the
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33 419 protein level is *INPP4B*, encoding for inositol polyphosphate-4-phosphatase type II B,
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35 420 a negative regulator of Akt.^{32,33} The expression of this phosphatase was upregulated in
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37 421 many cell types in tissues from achalasic patients, indicating a general increase in
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39 422 inhibitory signals for cell survival, a mechanism stimulated by Akt-dependent
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41 423 pathways.³² Thus, an *INPP4B* increased expression can be correlated to the
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43 424 enrichment in genes involved in cell death. Notably, in achalasia tissues we observed
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45 425 a decrease in the phosphorylation of the Ser473 in Akt, which is a key
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47 426 phosphorylation step for its activation and function. Therefore, it may be postulated
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49 427 that in achalasia the altered molecular signaling led to an overall impaired cell
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51 428 survival, which, in the long term, generates a permanent tissue damage. Thus, one can
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53 429 assume that also ICCs, in addition to neurons, can be affected. Since many studies
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3 430 have investigated the Akt / INPP4B pathway as a potential “druggable” route to block
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5 431 tumor proliferation,^{33,34} the development of *ad hoc* molecules may become relevant
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7 432 for patients with primary achalasia.
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11 434 In conclusion, our study highlights the altered expression of two distinct genes, i.e. c-
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13 435 KIT and INPP4B down- and up-regulation, in patients with primary achalasia. Our
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15 436 results may provide a basis for future research aimed at investigating novel
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17 437 therapeutic targets for patients with primary achalasia.
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19 438

20 439 **Acknowledgements**

21
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24 441 Gori, and V. Bertakis and Ms. G. Galiazzo for technical help.
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For Peer Review

532 **Table 1. Genes differentially expressed in achalasia.**

533 Genes overexpressed in achalasia are labeled in red. Green shadowed area indicates

534 the genes with a $P \leq 10^{-4}$.

ENSEMBL transcript ID	GENE name	log2 Fold Change	P value
ENST00000451137	CYR61	-2.99	9.71E-06
ENST00000367976	CTGF	-2.74	1.19E-05
ENST00000513000	INPP4B	1.63	1.42E-04
ENST00000288135	KIT	-1.48	4.17E-04
ENST00000249075	LIF	-2.40	4.23E-04
ENST00000239938	EGR1	-1.95	4.65E-04
ENST00000228434	CD69	-3.84	4.94E-04
ENST00000280979	AKAP6	1.63	7.28E-04
ENST00000296464	HSPA4L	1.07	8.99E-04
ENST00000319653	FMN2	2.03	1.11E-03
ENST00000517956	FBXO32	1.26	1.12E-03
ENST00000265634	NPTX2	-1.88	1.20E-03
ENST00000550683	ACVRL1	-1.14	1.25E-03
ENST00000378827	BMP2	-1.99	1.51E-03
ENST00000371852	CH25H	-3.06	1.56E-03
ENST00000284669	KLHL41	2.43	1.58E-03
ENST00000436924	BRE	2.70	1.60E-03
ENST00000561981	FRRS1L	1.51	1.62E-03
ENST00000307792	SEMA3E	1.07	1.66E-03
ENST00000330871	SOCS3	-2.47	2.07E-03
ENST00000331569	ZNF703	1.18	2.30E-03
ENST00000305988	ADRB2	-1.41	2.47E-03
ENST00000420022	ADM5	-1.80	2.58E-03
ENST00000297316	SOX17	-1.82	2.76E-03
ENST00000252590	PLVAP	-1.29	2.77E-03
ENST00000256103	PMP2	-2.28	2.87E-03
ENST00000329099	FAM101B	-1.32	3.11E-03
ENST00000287814	TIMP4	-1.25	3.12E-03
ENST00000300177	GREM1	-2.58	3.29E-03
ENST00000485685	GGNBP2	-1.78	3.40E-03
ENST00000365328	RN7SK	-1.61	3.41E-03
ENST00000284878	CXADR	2.20	3.47E-03
ENST00000296046	CPA3	-1.76	3.52E-03
ENST00000302754	JUNB	-1.73	3.74E-03
ENST00000437551	PAX8-AS1	1.36	3.74E-03
ENST00000322507	COL12A1	-1.10	3.80E-03
ENST00000307637	C3AR1	-1.86	3.82E-03
ENST00000340695	SCXA	2.40	3.88E-03
ENST00000322954	UACA	0.94	3.89E-03

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ENST0000055682	KIAA2022	1.00	3.92E-03
ENST00000379374	PHEX	-3.10	3.98E-03
ENST00000357949	SERTAD1	-1.45	4.02E-03
ENST00000230990	HBEGF	-2.36	4.08E-03
ENST00000359534	KCNK5	-2.25	4.16E-03
ENST00000224605	GDF10	-1.64	4.17E-03
ENST00000396184	PDE1C	1.02	4.32E-03
ENST00000373509	PIM1	-2.29	4.33E-03
ENST00000408965	CEBPD	-1.45	4.59E-03
ENST00000267984	MESDC1	-1.17	4.82E-03
ENST00000329608	CSF1	-1.16	4.92E-03
ENST00000344120	SPRY4	-1.18	5.13E-03
ENST00000395076	PPM1A	0.85	5.16E-03
ENST00000350896	MSR1	-1.37	5.21E-03
ENST00000366667	AGT	-1.68	5.22E-03
ENST00000263370	ITPKC	-1.75	5.24E-03
ENST00000259989	FGFBP2	-1.75	5.24E-03
ENST00000311922	TRIB1	-2.74	5.24E-03
ENST00000608521	MIR663A	2.80	5.25E-03
ENST00000377103	THBD	-2.31	5.27E-03
ENST00000422542	AC005682,5	-1.34	5.32E-03
ENST00000396037	ST8SIA1	1.75	5.39E-03
ENST00000264805	PDE5A	2.61	5.58E-03
ENST00000400546	NCAM2	-1.27	5.65E-03
ENST00000369026	MCL1	-1.16	6.00E-03
ENST00000515896	RNA5-8SP6	1.12	6.20E-03
ENST00000399799	ROCK1	0.96	6.23E-03
ENST00000273153	CSRNP1	-1.74	6.25E-03
ENST00000244221	PAIP2B	1.09	6.25E-03
ENST00000376414	GPR183	-1.77	6.40E-03
ENST00000500741	DYNLL1-AS1	1.16	6.57E-03
ENST00000308086	THAP2	0.91	6.67E-03
ENST00000274063	SFRP2	-1.92	6.68E-03
ENST00000290551	BTG2	-1.53	6.72E-03
ENST00000380392	C2CD4B	-2.57	6.78E-03
ENST00000410087	CERKL	-1.36	6.83E-03
ENST00000367558	RGS16	-2.28	7.09E-03
ENST00000256257	RNF122	-1.65	7.10E-03
ENST00000367511	FAM129A	0.64	7.12E-03
ENST00000592790	VMP1	-2.93	7.17E-03
ENST00000323040	GPR4	-1.97	7.27E-03
ENST00000307851	HAVCR2	-1.68	7.40E-03
ENST00000324559	ANO5	0.96	7.62E-03
ENST00000328041	SLC24A3	-1.35	7.63E-03
ENST00000311734	IL1RL1	-3.50	7.72E-03
ENST00000373313	MAFB	-1.65	8.05E-03

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ENST00000382723	MSX1	-2.11	8.32E-03
ENST00000379731	B4GALT1	-1.49	8.33E-03
ENST00000329399	PDLIM1	-1.46	8.42E-03
ENST00000369583	DUSP5	-1.58	8.43E-03
ENST00000332029	SOCS1	-1.69	8.47E-03
ENST00000441535	FMO2	-1.27	8.55E-03
ENST00000256951	EMP1	-2.33	8.71E-03
ENST00000239223	DUSP1	-1.08	8.75E-03
ENST00000330106	CEND1	-2.85	8.84E-03
ENST00000498165	PPM1L	1.08	8.87E-03
ENST00000506002	MTND6P4	1.03	8.91E-03
ENST00000244050	SNAI1	-1.59	8.93E-03
ENST00000380874	FOXC1	-1.77	8.94E-03
ENST00000396073	ENAM	2.83	9.03E-03
ENST00000272928	ACKR3	-1.31	9.04E-03
ENST00000379359	RGCC	-1.11	9.22E-03
ENST00000333926	CISD1	0.80	9.30E-03
ENST00000367996	ADAMTS4	-4.18	9.37E-03
ENST00000380079	STEAP4	-2.07	9.38E-03
ENST00000413366	PRKCA	1.03	9.39E-03
ENST00000215794	USP18	-1.45	9.57E-03
ENST00000305352	S1PR1	-1.27	9.74E-03
ENST00000306065	ANKRD27	0.67	9.81E-03
ENST00000222390	HGF	-2.02	9.83E-03
ENST00000358432	EPHA2	-1.13	9.85E-03
ENST00000270001	ZFP14	0.88	9.89E-03

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536

537 **Table 2.** Output of PANTHER Over-representation test of biological processes
 538 enriched for the genes differentially expressed in achalasia tissues, compared to H.
 539 sapiens gene PANTHER database.
 540

PANTHER GO Slim Biological process	<i>H. sapiens</i>	Achalasia	Expected	Fold enrichment	Hierarchy (+/-)	raw <i>P</i> value	False Discovery Rate
Protein phosphorylation	81	4	.42	9.53	+	9.80e-04	3.99e-02
MAPK cascade	340	9	1.76	5.11	+	8.19e-05	6.66e-03
• intracellular signal transduction	1071	17	5.55	3.06	+	4.05e-05	4.94e-03
• signal transduction	2318	24	12.01	2.00	+	1.01e-03	3.54e-02
• Cell communication	2686	26	13.91	1.87	+	1.39e-03	3.76e-02
Regulation of catalytic activity	359	9	1.86	4.84	+	1.22e-04	7.47e-03
• regulation of molecular function	441	9	2.28	3.94	+	5.38e-04	2.62e-02
Regulation of phosphate metabolic process	537	12	2.78	4.31	+	2.64e-05	6.44e-03
Developmental process	1501	18	7.78	2.32	+	1.03e-03	3.13e-02

541
 542 Only results with False Discovery Rate<0.05 are reported in the table.
 543

544 **Table 3:** Output of STRING (protein-protein interaction and biological processes) for
 545 genes differentially regulated from whole transcriptome analysis of achalasia tissues.
 546

	Pathway ID and description	Count in gene set	False Discovery Rate <i>P</i> values
Biological Process (GO)	GO 0051094 positive regulation of developmental process	29	1.97e-10
	GO 1901342 regulation of vascular development	15	8.62e-10
	GO 0045597 positive regulation of cell differentiation	24	1.44e-09
	GO 004565 regulation of angiogenesis	14	2.7e-09
	GO 2000026 regulation of multicellular organismal development	31	3.31e-09
Molecular function (GO)	GO 0001228 transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding	9	0.0483
	GO 0005515 protein binding	40	0.0483
	GO 0008083 growth factor activity	3	0.0483
Cellular component (GO)	GO 005615 extracellular space	20	0.00342
	GO 0009986 cell surface	14	0.00552

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 548 Statistical background selected for the enrichment analysis set to “whole genome”.
 549

549 Figure Legends

550

551 **Figure 1. Differentially expressed genes in achalasia tissue.** (A) Heat map showing
552 the concordant differential expression observed in the tissues from achalasia patients
553 compared to controls (for the genes reported in Table 1). Elaborated with MeV v4.8.1.
554 (B) Biological processes of genes (GO-Slim Biological processes) differentially
555 expressed in achalasia, as identified by PANTHER Functional Classification analysis.
556 (C) Graphical output of significantly enriched protein-protein interactions determined
557 with STRING analysis, showing the results of the 104 out of 111 dysregulated genes
558 in achalasia, compared with data present in the STRING databases. The different
559 colors in the figure inset identify the evidence for the different interactions (known;
560 predicted; identified with other means, such as co-expression, data mining, and
561 protein homology). The red square indicates c-KIT. (D) Real-time qRT-PCR data for
562 *c-KIT* and *INPP4B*. Data from case and control tissue biopsies were normalized on a
563 commercial pool of control tissue biopsies from esophagus. Student's t-test was
564 performed and *P* values are indicated. Bars indicate standard deviations.

565

566 **Figure 2. Western blot analysis of differentially expressed genes.** (A) Example of
567 the protein expression profiles in achalasia tissues compared to control samples for c-
568 KIT and INPP4B. Western blotting was repeated in at least two independent
569 experiments. (B) Densitometric analysis of the relative intensities for c-KIT and
570 INPP4B. Quantitative data (mean values \pm standard deviation) from different
571 experiments obtained from each individual were compared using Student's t-test for
572 statistical analysis. For each sample, densitometric values were normalized on
573 reference protein (vinculin). All experiments were carried out at least twice. Bars
574 indicated standard deviations. *P* values are indicated in the graphs.

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5 576 **Figure 3. Immunostaining for c-KIT (A) and INPP4B (B).** (A) Examples of the
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7 577 expression pattern for c-KIT in tissue biopsies from control (left) and achalasia
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9 578 patients (right). Arrows indicate the positive staining for c-KIT. c-KIT staining color
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11 579 histograms of controls (n=6) and cases (n=18) represent the number of samples
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13 580 assessed according to the semi-quantitative score. c-KIT immunolabeling differences
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15 581 in cases vs. controls were assessed by Fisher's exact test. (B) Examples of the
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17 582 expression pattern for INPP4B in tissue biopsies from control (left) and achalasia
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19 583 patients (right). INPP4B staining color histograms of controls (n=6) and cases (n=22)
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21 584 represent the number of samples assessed according to the semi-quantitative score.
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23 585 INPP4B immunolabeling differences in cases vs. controls were assessed by Fisher's
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25 586 exact test. Scale bars are 100 μ m as indicated.
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31 588 **Figure 4. Phospho-Akt is down regulated in achalasia.** (A) Example of the protein
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33 589 expression profiles in achalasia tissues compared to control samples of phospho-Akt
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35 590 (Ser473; Thr308) and total Akt. (B) Quantitative data (mean values \pm standard
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37 591 deviation) from different experiments (performed in duplicate) obtained from each
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39 592 individual were compared using Student's t-test. For each sample, densitometric
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41 593 values were normalized on reference protein (β -actin). The significantly different
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43 594 relative phospho-Akt (Ser473) levels comparing achalasia vs. control tissues, were
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45 595 reported in the histogram (P=0.0495, Student's t-test). Bars indicate standard
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47 596 deviations.
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Supplementary Table 1: Clinical and epidemiological data of achalasia patients (a) and controls (b)

a) Achalasia

ACHA pts ID	Sex	Age	Age at diagnosis	LES basal pressure (mm Hg)	Duration of symptoms before diagnosis(yrs)	Type of molecular analysis
ITA_ACHA77	M	43	30	15	15	wb
ITA_ACHA69	M	84	83	53	20	wb
ITA_ACHA74	M	42	41	22.5	5	wb
ITA_ACHA59	F	51	50	20	3	wb/IHC
ITA_ACHA81	F	24	20	18	5	wb
ITA_ACHA40	F	42	39	28	8	wb
ITA_ACHA17	F	46	45	20	10	wb
ITA_ACHA76	M	55	54	24	10	wb
ITA_ACHA71	F	72	70	15	8	wb
ITA_ACHA34	M	68	67	44	8	RNAseq
ITA_ACHA16	F	25	24	18	2	RNAseq
ITA_ACHA75	F	75	58	15	25	IHC
ITA_ACHA37	F	64	61	18	4	RNAseq/RT-qPCR/wb/IHC
ITA_ACHA34	M	58	57	35	20	/wb
ITA_ACHA02	F	37	34	25	4	wb/IHC
ITA_ACHA73	M	81	78	28	4	wb
ITA_ACHA33	M	68	67	44	2	RNAseq/RT-qPCR/wb/IHC
ITA_ACHA25	M	66	64	22	3	wb
ITA_ACHA78	F	36	36	15	1	IHC
ITA_ACHA66	F	41	40	23	20	wb
BE_ACHA5051	F	16	16	37	6	IHC
BE_ACHA5208	M	69	69	na ^{a)}	7	IHC
BE_ACHA5691	F	82	82	na	2.5	IHC
BE_ACHA6936	F	20	20	38	2	IHC
BE_ACHA7521	F	63	63	35.3	0.42	IHC
BE_ACHA5466	M	22	22	27.5	1	IHC
BE_ACHA5768	M	44	44	33	1.5	IHC
BE_ACHA6790	F	69	69	35.6	2	IHC
BE_ACHA810	M	61	61	30	15	IHC
BE_ACHA8705	M	65	65	na	10	IHC
BE_ACHA1059	M	28	28	na ^{a)}	7	IHC
BE_ACHA8154	F	42	42	68	0.58	IHC
BE_ACHA302	M	50	50	51	3	IHC
BE_ACHA479	M	50	50	34	3	IHC
BE_ACHA7818	F	33	33	38	0.17	IHC
BE_ACHA8199	M	24	24	103	6	IHC
BE_ACHA8347	F	21	21	na ^{b)}	4	IHC

BE_ACHA5260	F	32	32	48.3	2	IHC
BE_ACHA5295	F	35	35	na	6	IHC
BE_ACHA1730	M	57	57	nm	5	IHC
BE_ACHA3017	M	49	49	42.3	2	IHC
BE_ACHA3354	F	49	49	80.3	8	IHC

Legends: na= not available; nm= not measurable; ^{a)} type I; ^{b)} type II; wb= western blotting; IHC= immunohistochemistry.

b) controls

ID patients	Age	Sex	Pathology	Surgery	Histology	Type of molecular analysis
ITA_CNT_01	64	F	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	IHC
ITA_CNT_02	45	M	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	IHC
ITA_CNT_03	77	M	Esophageal adenocarcinoma - Siewert II	Total gastrectomy and resection of the distal esophagus (10 cm section above the diaphragmatic hiatus)	Intestinal adenocarcinoma (T3-N1-M0). Tumor-free margins	wb/IHC
ITA_CNT_70	55	M	Megaesophagus	Partial esophagectomy with gastro-esophageal reconstruction		IHC
ITA_CNT_72	87	M	Esophageal adenocarcinoma - Siewert I	Partial esophagectomy with gastro-esophageal reconstruction	Intestinal adenocarcinoma (T2-N1-M0). Tumor-free margins	IHC
ITA_CNT_04	69	M	Esophageal adenocarcinoma - Siewert II	Total gastrectomy and resection of the distal esophagus (10 cm section above the diaphragmatic hiatus)	Intestinal adenocarcinoma (T4-N3-M0). Tumor-free margins	IHC
ITA_CNT_26	49	F	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	RNAseq/wb
ITA_CNT_23	46	F	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	RNAseq/wb/IHC
ITA_CNT_41	66	M	Esophageal adenocarcinoma - Siewert I	Partial esophagectomy with gastro-esophageal reconstruction	Intestinal adenocarcinoma (T2-N1-M0). Tumor-free margins	RNAseq/wb
ITA_CNT_52	57	M	Esophageal adenocarcinoma - Siewert I	Partial esophagectomy with gastro-esophageal reconstruction	Intestinal adenocarcinoma (T3-N3-M0). Tumor-free margins	RNAseq/RT-qPCR/wb/IHC

Supplementary Table S2. Total read number and mean coverage of RNAseq libraries.

Sample ID	Total read number	Mean coverage (X)
ITA_ACHA16	96,763,286	63
ITA_ACHA34	79,986,036	43
ITA_ACHA37	85,671,948	47
ITA_ACHA33	74,486,236	55
ITA_CNT29	70,830,832	36
ITA_CNT26	70,217,988	55
ITA_CNT41	83,327,050	64
ITA_CNT52	81,841,600	68

Supplementary Table 1: Clinical and epidemiological data of achalasia patients (a) and controls (b)

a) Achalasia

ACHA pts ID	Sex	Age	Age at diagnosis	LES basal pressure (mm Hg)	Duration of symptoms before diagnosis(yrs)	Type of molecular analysis
ITA_ACHA77	M	43	30	15	15	wb
ITA_ACHA69	M	84	83	53	20	wb
ITA_ACHA74	M	42	41	22.5	5	wb
ITA_ACHA59	F	51	50	20	3	wb/IHC
ITA_ACHA81	F	24	20	18	5	wb
ITA_ACHA40	F	42	39	28	8	wb
ITA_ACHA17	F	46	45	20	10	wb
ITA_ACHA76	M	55	54	24	10	wb
ITA_ACHA71	F	72	70	15	8	wb
ITA_ACHA34	M	68	67	44	8	RNAseq
ITA_ACHA16	F	25	24	18	2	RNAseq
ITA_ACHA75	F	75	58	15	25	IHC
ITA_ACHA37	F	64	61	18	4	RNAseq/RT-qPCR/wb/IHC
ITA_ACHA34	M	58	57	35	20	/wb
ITA_ACHA02	F	37	34	25	4	wb/IHC
ITA_ACHA73	M	81	78	28	4	wb
ITA_ACHA33	M	68	67	44	2	RNAseq/RT-qPCR/wb/IHC
ITA_ACHA25	M	66	64	22	3	wb
ITA_ACHA78	F	36	36	15	1	IHC
ITA_ACHA66	F	41	40	23	20	wb
BE_ACHA5051	F	16	16	37	6	IHC
BE_ACHA5208	M	69	69	na ^{a)}	7	IHC
BE_ACHA5691	F	82	82	na	2.5	IHC
BE_ACHA6936	F	20	20	38	2	IHC
BE_ACHA7521	F	63	63	35.3	0.42	IHC
BE_ACHA5466	M	22	22	27.5	1	IHC
BE_ACHA5768	M	44	44	33	1.5	IHC
BE_ACHA6790	F	69	69	35.6	2	IHC
BE_ACHA810	M	61	61	30	15	IHC
BE_ACHA8705	M	65	65	na	10	IHC
BE_ACHA1059	M	28	28	na ^{a)}	7	IHC
BE_ACHA8154	F	42	42	68	0.58	IHC
BE_ACHA302	M	50	50	51	3	IHC
BE_ACHA479	M	50	50	34	3	IHC
BE_ACHA7818	F	33	33	38	0.17	IHC
BE_ACHA8199	M	24	24	103	6	IHC
BE_ACHA8347	F	21	21	na ^{b)}	4	IHC

BE_ACHA5260	F	32	32	48.3	2	IHC
BE_ACHA5295	F	35	35	na	6	IHC
BE_ACHA1730	M	57	57	nm	5	IHC
BE_ACHA3017	M	49	49	42.3	2	IHC
BE_ACHA3354	F	49	49	80.3	8	IHC

Legends: na= not available; nm= not measurable; ^{a)} type I; ^{b)} type II; wb= western blotting; IHC= immunohistochemistry.

b) controls

ID patients	Age	Sex	Pathology	Surgery	Histology	Type of molecular analysis
ITA_CNT_01	64	F	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	IHC
ITA_CNT_02	45	M	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	IHC
ITA_CNT_03	77	M	Esophageal adenocarcinoma - Siewert II	Total gastrectomy and resection of the distal esophagus (10 cm section above the diaphragmatic hiatus)	Intestinal adenocarcinoma (T3-N1-M0). Tumor-free margins	wb/IHC
ITA_CNT_70	55	M	Megaesophagus	Partial esophagectomy with gastro-esophageal reconstruction		IHC
ITA_CNT_72	87	M	Esophageal adenocarcinoma - Siewert I	Partial esophagectomy with gastro-esophageal reconstruction	Intestinal adenocarcinoma (T2-N1-M0). Tumor-free margins	IHC
ITA_CNT_04	69	M	Esophageal adenocarcinoma - Siewert II	Total gastrectomy and resection of the distal esophagus (10 cm section above the diaphragmatic hiatus)	Intestinal adenocarcinoma (T4-N3-M0). Tumor-free margins	IHC
ITA_CNT_26	49	F	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	RNAseq/wb
ITA_CNT_23	46	F	Gastroesophageal reflux	Antireflux Nissen fundoplication	-	RNAseq/wb/IHC
ITA_CNT_41	66	M	Esophageal adenocarcinoma - Siewert I	Partial esophagectomy with gastro-esophageal reconstruction	Intestinal adenocarcinoma (T2-N1-M0). Tumor-free margins	RNAseq/wb
ITA_CNT_52	57	M	Esophageal adenocarcinoma - Siewert I	Partial esophagectomy with gastro-esophageal reconstruction	Intestinal adenocarcinoma (T3-N3-M0). Tumor-free margins	RNAseq/RT-qPCR/wb/IHC

Supplementary Table S2. Total read number and mean coverage of RNAseq libraries.

Sample ID	Total read number	Mean coverage (X)
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ITA_CNT26	70,217,988	55
ITA_CNT41	83,327,050	64
ITA_CNT52	81,841,600	68

Supporting Material for reviewers Only

New! PANTHER13.1 released.

Analysis Summary: Please report in publication [?](#)

Analysis Type: PANTHER Overrepresentation Test (Released 20171205)	
Annotation Version and Release Date: PANTHER version 13.1 Released 2018-02-03	
Analyzed List:	Client Text Box Input (Homo sapiens) Change
Reference List:	Homo sapiens (all genes in database) Change
Annotation Data Set:	PANTHER GO-Slim Biological Process ▼
Test Type:	<input checked="" type="radio"/> Fisher's Exact with FDR multiple test correction <input type="radio"/> Binomial

Results [?](#)

	Reference list	Client Text Box Input
Mapped IDs:	21042 out of 21042	105 out of 109
Unmapped IDs:	0	7
Multiple mapping information:	0	4

[Export results](#) View: [-- Please select a chart to display --](#) [▼](#)

Displaying only results with False Discovery Rate < 0.05; [click here to display all results](#)

	Homo sapiens (REF)	Client Text Box Input (▼ Hierarchy NEW! ?)					
	#	#	expected	Fold Enrichment	+/-	raw P value	FDR
PANTHER GO-Slim Biological Process							
protein phosphorylation	81	4	0.42	9.53	+	9.80E-04	3.99E-02
MAPK cascade	340	9	1.76	5.11	+	8.19E-05	6.66E-03
↳intracellular signal transduction	1071	17	5.55	3.06	+	4.05E-05	4.94E-03
↳signal transduction	2318	24	12.01	2.00	+	1.01E-03	3.54E-02
↳cell communication	2686	26	13.91	1.87	+	1.39E-03	3.76E-02
regulation of catalytic activity	359	9	1.86	4.84	+	1.22E-04	7.47E-03
↳regulation of molecular function	441	9	2.28	3.94	+	5.38E-04	2.62E-02
regulation of phosphate metabolic process	537	12	2.78	4.31	+	2.64E-05	6.44E-03
developmental process	1501	18	7.78	2.32	+	1.03E-03	3.13E-02

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For Peer Review

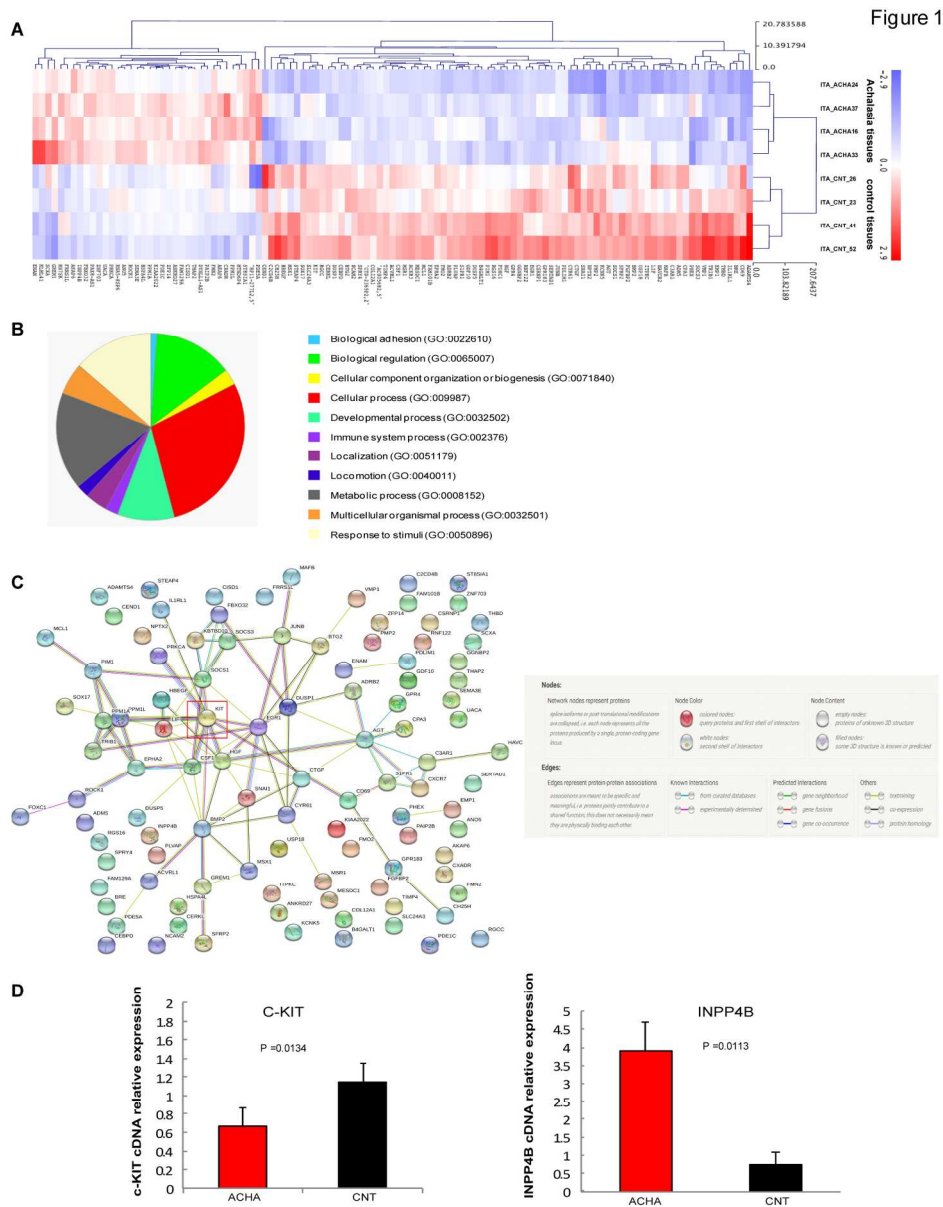


Figure 1. Differentially expressed genes in achalasia tissue. (A) Heat map showing the concordant differential expression observed in the tissues from achalasia patients compared to controls (for the genes reported in Table 1). Elaborated with MeV v4.8.1. (B) Biological processes of genes (GO-Slim Biological processes) differentially expressed in achalasia, as identified by PANTHER Functional Classification analysis. (C) Graphical output of significantly enriched protein-protein interactions determined with STRING analysis, showing the results of the 104 out of 111 dysregulated genes in achalasia, compared with data present in the STRING databases. The different colors in the figure inset identify the evidence for the different interactions (known; predicted; identified with other means, such as co-expression, data mining, and protein homology). The red square indicates c-KIT. (D) Real-time qRT-PCR data for c-KIT and INPP4B. Data from case and control tissue biopsies were normalized on a commercial pool of control tissue biopsies from esophagus. Student's t-test was performed and P values are indicated. Bars indicate standard deviations.

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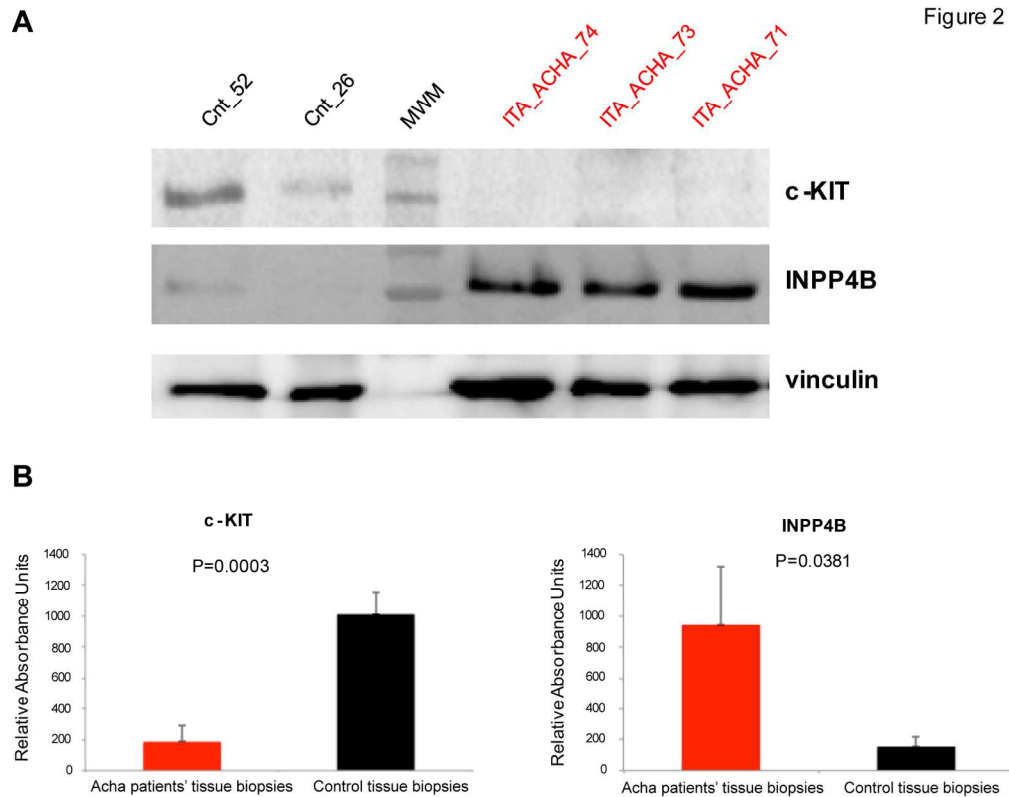


Figure 2. Western blot analysis of differentially expressed genes. (A) Example of the protein expression profiles in achalasia tissues compared to control samples for c-KIT and INPP4B. Western blotting was repeated in at least two independent experiments. (B) Densitometric analysis of the relative intensities for c-KIT and INPP4B. Quantitative data (mean values \pm standard deviation) from different experiments obtained from each individual were compared using Student's t-test for statistical analysis. For each sample, densitometric values were normalized on reference protein (vinculin). All experiments were carried out at least twice. Bars indicated standard deviations. P values are indicated in the graphs.

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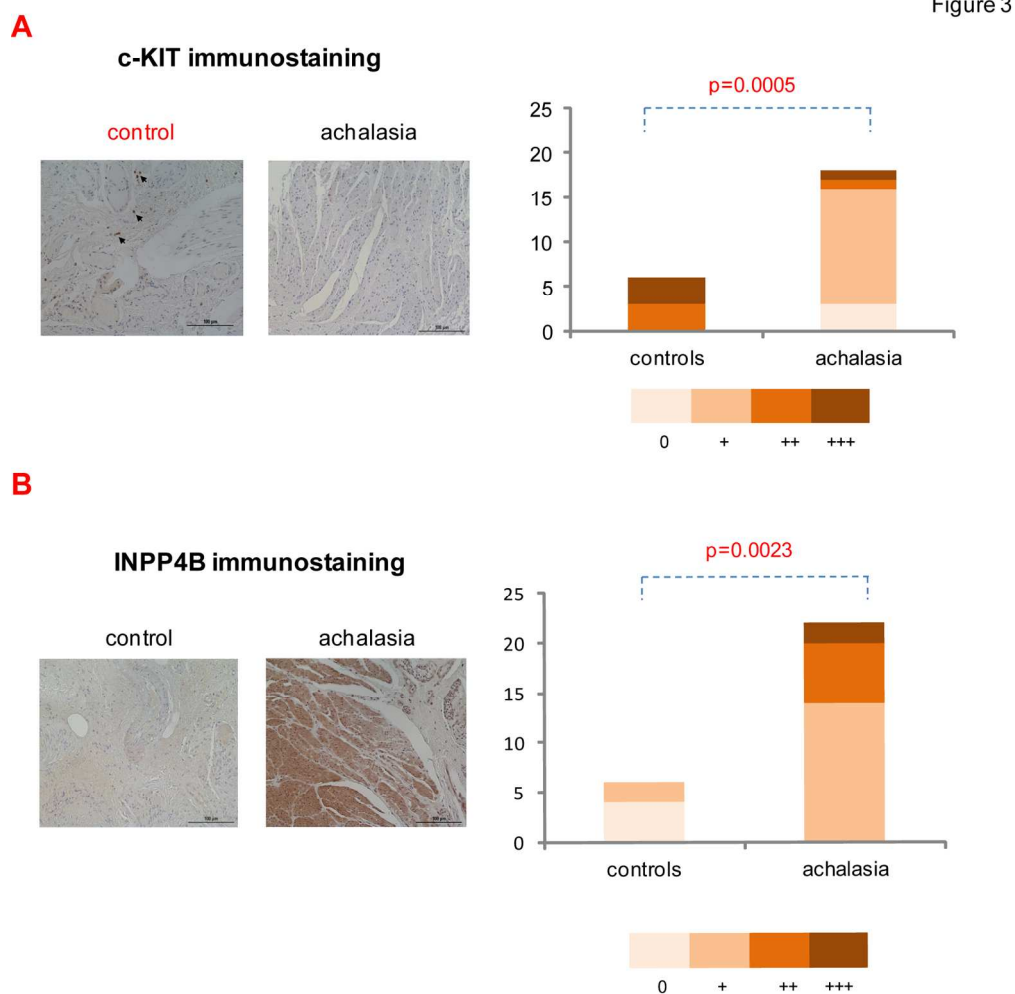


Figure 3. Immunostaining for c-KIT (A) and INPP4B (B). (A) Examples of the expression pattern for c-KIT in tissue biopsies from control (left) and achalasia patients (right). Arrows indicate the positive staining for c-KIT. c-KIT staining color histograms of controls (n=6) and cases (n=18) represent the number of samples assessed according to the semi-quantitative score. c-KIT immunolabeling differences in cases vs. controls were assessed by Fisher's exact test. (B) Examples of the expression pattern for INPP4B in tissue biopsies from control (left) and achalasia patients (right). INPP4B staining color histograms of controls (n=6) and cases (n=22) represent the number of samples assessed according to the semi-quantitative score. INPP4B immunolabeling differences in cases vs. controls were assessed by Fisher's exact test. Scale bars are 100 μ m as indicated.

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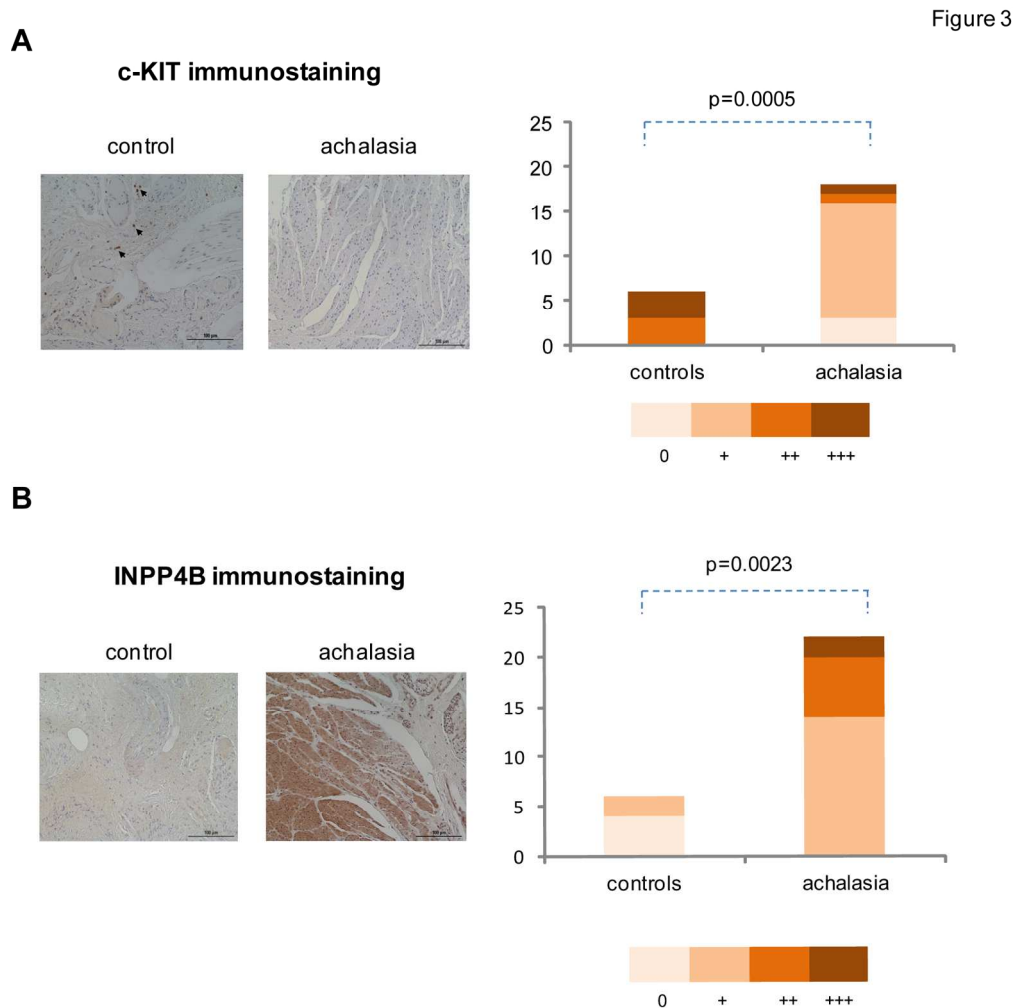


Figure 3. Immunostaining for c-KIT (A) and INPP4B (B). (A) Examples of the expression pattern for c-KIT in tissue biopsies from control (left) and achalasia patients (right). Arrows indicate the positive staining for c-KIT. c-KIT staining color histograms of controls (n=6) and cases (n=18) represent the number of samples assessed according to the semi-quantitative score. c-KIT immunolabeling differences in cases vs. controls were assessed by Fisher's exact test. (B) Examples of the expression pattern for INPP4B in tissue biopsies from control (left) and achalasia patients (right). INPP4B staining color histograms of controls (n=6) and cases (n=22) represent the number of samples assessed according to the semi-quantitative score. INPP4B immunolabeling differences in cases vs. controls were assessed by Fisher's exact test. Scale bars are 100µm as indicated.

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Figure 4

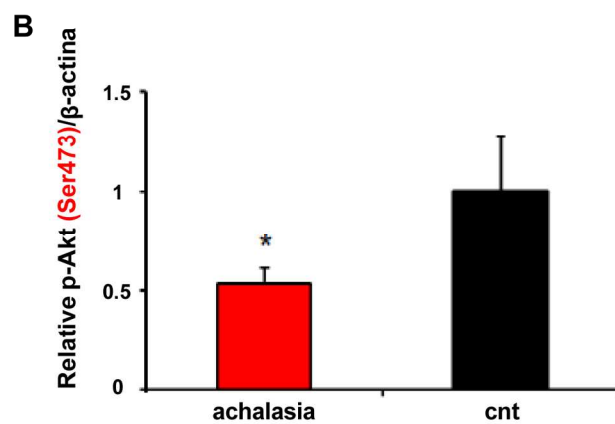
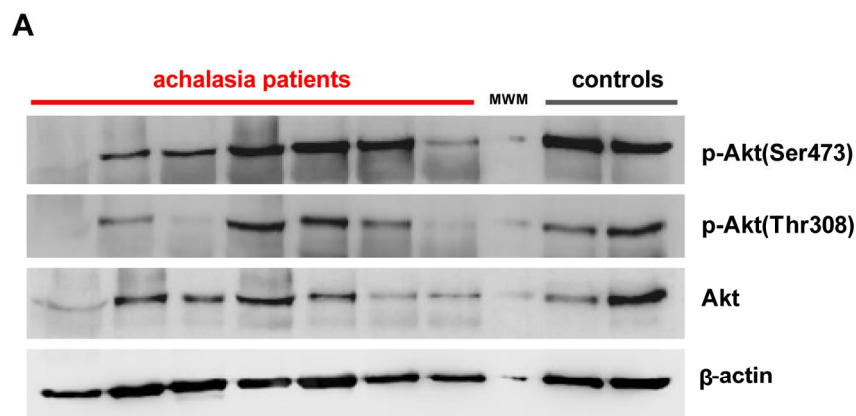


Figure 4. Phospho-Akt is down regulated in achalasia. (A) Example of the protein expression profiles in achalasia tissues compared to control samples of phospho-Akt (Ser473; Thr308) and total Akt. (B) Quantitative data (mean values \pm standard deviation) from different experiments (performed in duplicate) obtained from each individual were compared using Student's t-test. For each sample, densitometric values were normalized on reference protein (β -actin). The significantly different relative phospho-Akt (Ser473) levels comparing achalasia vs. control tissues, were reported in the histogram ($P=0.0495$, Student's t-test). Bars indicate standard deviations.

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Figure 4

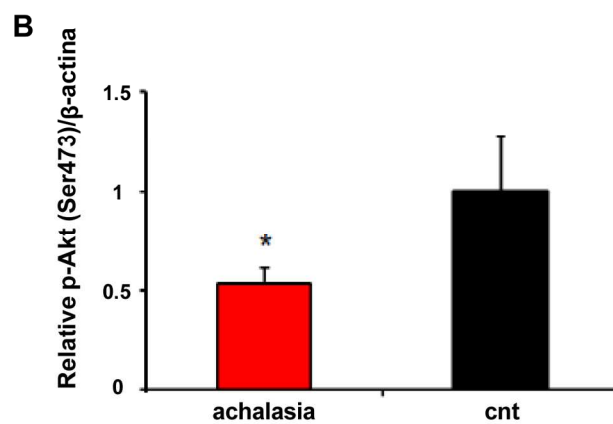
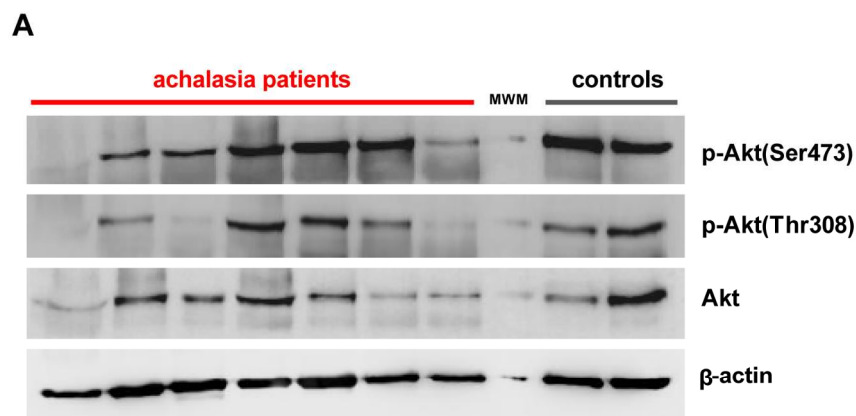


Figure 4. Phospho-Akt is down regulated in achalasia. (A) Example of the protein expression profiles in achalasia tissues compared to control samples of phospho-Akt (Ser473; Thr308) and total Akt. (B) Quantitative data (mean values \pm standard deviation) from different experiments (performed in duplicate) obtained from each individual were compared using Student's t-test. For each sample, densitometric values were normalized on reference protein (β -actin). The significantly different relative phospho-Akt (Ser473) levels comparing achalasia vs. control tissues, were reported in the histogram ($P=0.0495$, Student's t-test). Bars indicate standard deviations.

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