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# Η ΥΠΕΡΕΚΦΡΑΣΗ ΤΟΥ *SMAD7* ΠΡΟΣΤΑΤΕΥΕΙ ΤΟ ΗΠΑΡ ΑΠΟ ΤΗΝ TGF-β/SMAD ΜΕΣΟΛΑΒΟΥΜΕΝΗ ΙΝΟΓΕΝΕΣΗ

## Overexpression of SMAD7 protects liver from TGF-β/Smad-mediated fibrogenesis

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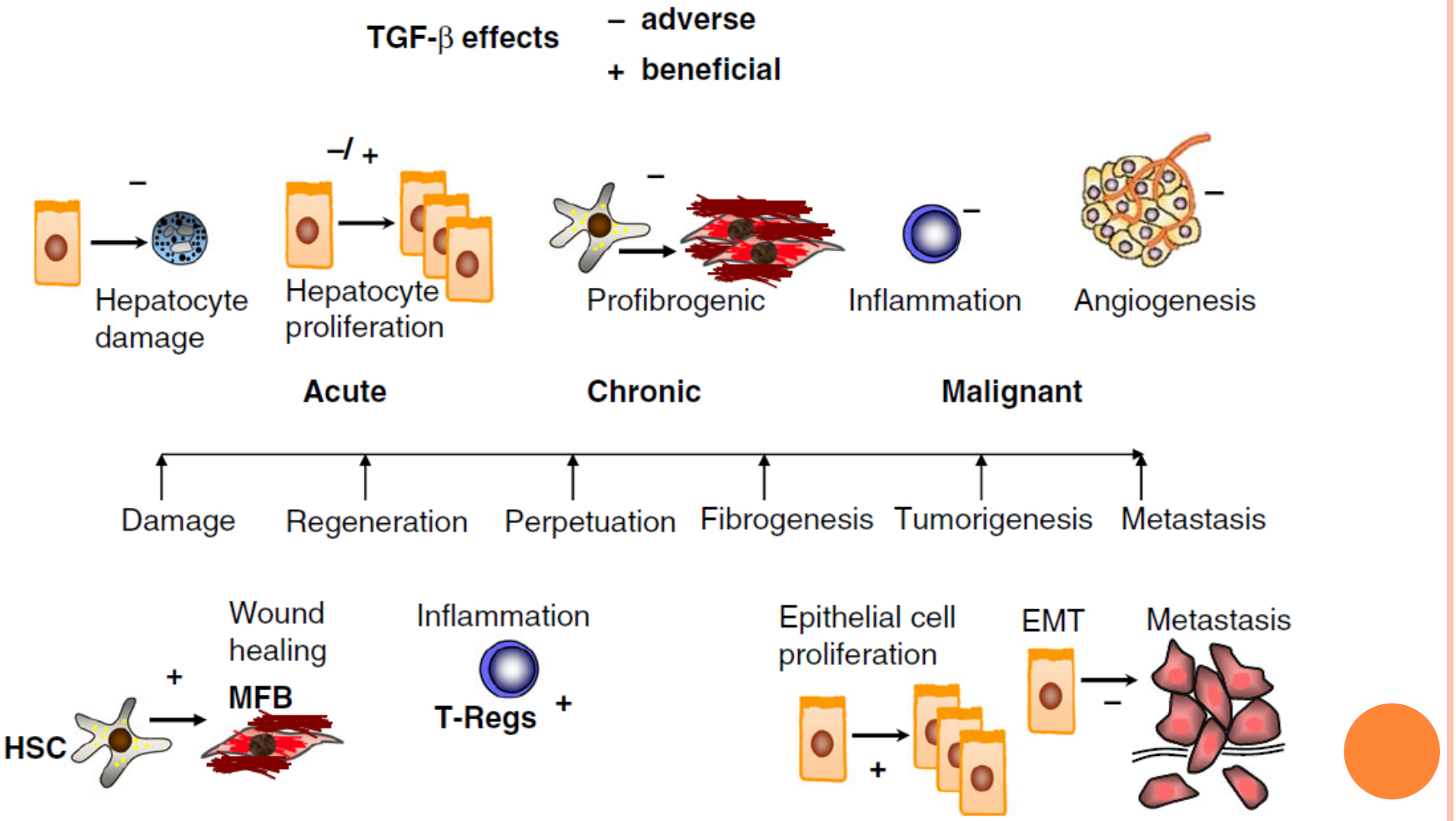
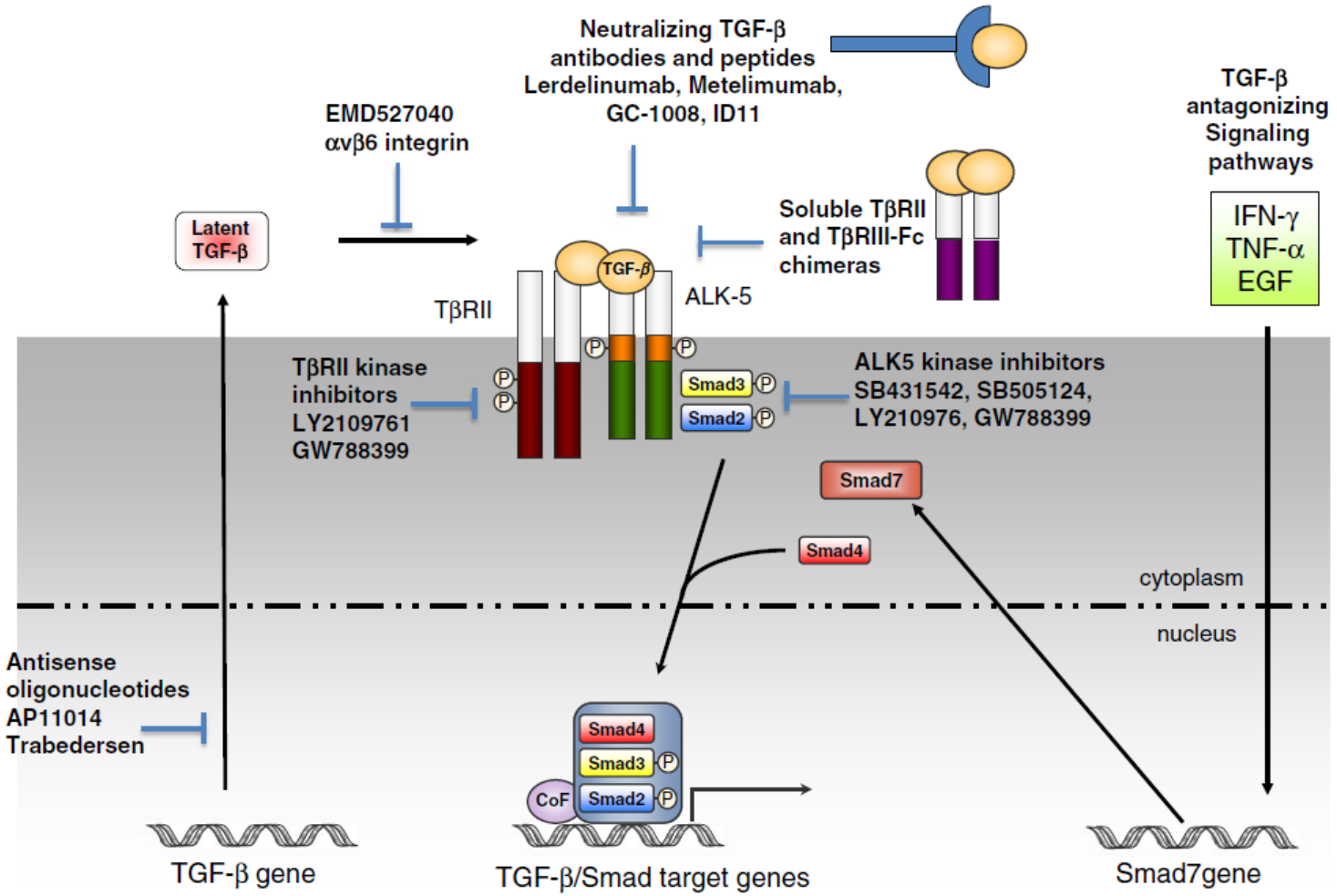
# Pros and cons of transforming growth factor-b (TGF-b) signalling during the progression of chronic liver diseases

Cell Tissue Res (2012) 347:245–256



# TGF-B SIGNAL TRANSDUCTION PATHWAY AND TARGETS FOR THERAPEUTIC INTERVENTION

CELL TISSUE RES (2012) 347:245–256



# Background

- Smad7 is a dominant intracellular inhibitor of TGFb/Activin signal transduction.[1] Recent animal model studies have corroborated the protective function of Smad7 in attenuating TGF-β-mediated fibrosis in multiple organs, including liver, through manipulation of Smad7 expression.[2-5]
- This study was scheduled in order to determine whether *Smad7* mRNA expression correlates with the expression of the molecules participating in the TGF-β/Activin signal transduction pathway in liver tissue of patients with chronic hepatic diseases and to seek correlations with the status of liver inflammation, fibrosis and the effect of treatment.

# Patients & Methods(1)

- **Liver biopsies** obtained from **67 patients with chronic hepatic diseases** including
- a) 18 with chronic HCV hepatitis (CHC);
- b) 19 with chronic HBV hepatitis at diagnosis (CHB/d);
- c) 4 with CHB after antiviral treatment and relapse (CHB/nr)
- d) 14 with CHB after antiviral treatment response and maintained remission for >5y (CHB/r);
- e) 12 with non alcoholic fatty liver disease (NAFLD).
- Three individuals submitted to liver biopsy due to a mild increase of aminotransferases but without liver architecture changes (served as controls).
- Demographic, clinicopathological and serological data of the analyzed subjects are summarized in **Table 1**.

Table 1. Clinicopathological and serological data of the patients of the study

| | Controls | CHB/d [a] | CHB/nr [b] | CHB/r [c] | CHC [d] | NAFLD [e] |
|---|---|---|---|---|---|---|
| **No** | 3 | 19 | 4 | 14 | 18 | 12 |
| **Sex (M/F) [f]** | 2/1 | 9/10 | 2/2 | 11/3 | 14/4 | 7/5 |
| **Age (median, range)** | 61, 60-67 | 54, 24-64 | 57, 22-65 | 52, 23-60 | 41.5, 27-54 | 45, 21-71 |
| **AST [g] (U/μL), (median, range)** | 42, 36-45 | 51, 17-1969 | 62, 39-277 | 29.5, 15-51 | 45, 24-237 | 31.5, 19-70 |
| **ALT [h] (U/μL), (median, range)** | 32, 21-48 | 61, 15-1478 | 97.5, 70-332 | 31.5, 17-49 | 75, 32-213 | 54, 15-141 |
| **Inflammation grade [i]** | | | | | | |
| **I-0 [i]** | 3 | — | — | 1 | — | 3 |
| **I-1 [i]** | — | 4 | — | 10 | 2 | 4 |
| **I-2 [i]** | — | 8 | 3 | 3 | 10 | 5 |
| **I-3 [i]** | — | 5 | 1 | — | 6 | — |
| **I-4 [i]** | — | 2 | — | — | — | — |
| **Fibrosis (median, range)[i]** | — | 4.0, 0-6 | 4.5, 1-5 | 2.0, 0-3 | 3.0, 1-6 | 0.5, 0-2 |
| **HAI-score (median, range)** | — | 8.0, 1-15 | 8.0, 5-11 | 2.0, 0-7 | 7.0, 2-12 | 2.0, 0-5 |
| **Viral load (median, range)** | — | 4 Meq/mL (0.009-699) | 0.10 Meq/mL (0-44.5) | 0 Meq/mL (0-0.008) | 0.70 Meq/mL (0.10-6.25) | 0, 0-0 |

**Abbreviations:** [a] CHB/d, newly diagnosed patients with Chronic HBV hepatitis; [b] CHB/nr, CHB patients 6 months after treatment withdrawal and no virologic/biochemical sustained response, [c] CHB/r, CHB patients after antiviral treatment response and remission for >5y, [d] CHC, Chronic HCV hepatitis; [e] NAFLD, non-alcoholic fatty liver disease; [f] M, male; F, female; [g] AST, aspartate aminotransferase; [h] ALT, alanine aminotransferase; [i] Inflammation grade (I-0: without inflammation, I-1: minimal, I-2: mild, I-3: moderate and I- 4: marked) and fibrosis

# Patients & Methods(2)

- The **mRNA levels** of
- ***TGFBs (TGFB-1,-2,-3*)**,
- **activins (*A,B,C,E*)**, ***ALK4*, *ALK5*,**
- **SMAD molecules (*SMAD-2, -3, -4, -7*),** and
- ***CTGF*** were determined in a quantitative reverse transcriptase PCR using SYBR-Green PCR Supermix (Invitrogen,UK).
- Primers were either designed or commercially obtained by SA Biosciences (USA). The sequences of the designed primers as well as the thermocycling conditions for all genes are summarized in **Table 2**.
- The *beta-2-microglobulin* **(*B2M*)** gene was used as a reference gene for sample normalization.
- Statistical analyses were performed using the SPSS ver. 18.0 software.

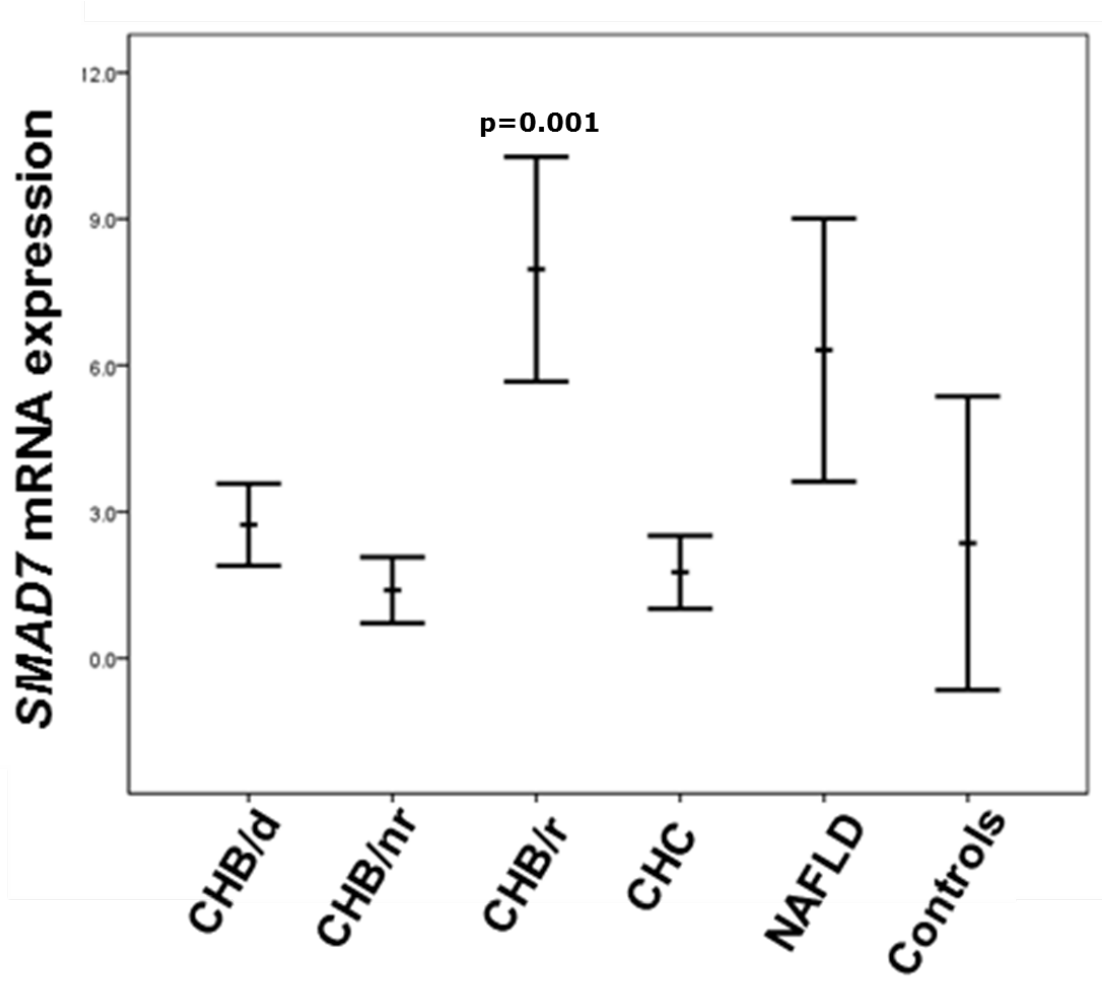
Table 2. Primers and PCR conditions for the amplification of the analyzed genes

| Gene | Primers | Sequence | PCR conditions |
|---|---|---|---|
| ***TGFB1*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH00508A | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 60$^0$C for 60 s) |
| ***TGFB2*** | forward<br>reverse | 5'- AgAgTgCCTgAACAA -3'<br>5'- CCATTCgCCTTCTgCTCTT -3' | 95$^0$C for 2 min, followed by 40 cycles<br>(95$^0$C for 15 s, 53$^0$C for 15 s, 72$^0$C for 15 s) |
| ***TGFB3*** | forward<br>reverse | commercially obtained by SABiosciences ,<br>Cat No PPH00531E | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 10 s, 58$^0$C for 10 s, 72$^0$C for 30 s) |
| ***ALK5*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH00237B | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 60$^0$C for 60 s) |
| ***ALK4*** | forward<br>reverse | 5'- CATTgACATTgCCCCgAATC -3'<br>5'- CgAgCAATCTCCCAATATACAAg -3' | 95$^0$C for 2 min, followed by 50 cycles<br>(95$^0$C for 15 s, 56$^0$C for 40 s), and 72$^0$C for 2 min |
| ***SMAD2*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH01949E | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 58$^0$C for 15 s, 72$^0$C for 15 s) |
| ***SMAD3*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH01921B | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 10 s, 58$^0$C for 10 s, 72$^0$C for 30 s) |
| ***SMAD4*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH00134B | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 60$^0$C for 60 s) |
| ***SMAD7*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH01905B | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 60$^0$C for 60 s) |
| ***CTGF*** | forward<br>reverse | 5'- ACCAATgACAACgCCTCCTg -3'<br>5'- TTgCCCTTCTTA ATgTTCTCTTCC -3' | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 60$^0$C for 60 s) |
| ***INHBA*** | forward<br>reverse | 5'- AgCAgACCTCggAgATCATC -3'<br>5'- TTggggACTTTTAggAAgAgC -3' | 95$^0$C for 2 min, followed by 50 cycles<br>(95$^0$C for 15 s, 56$^0$C for 40 s), and 72$^0$C for 2 min |
| ***INHBB*** | forward<br>reverse | 5'- AggAgCgCgTTTCCgAAATC -3'<br>5'- TggTTgCCTTCgTTggAgATg -3' | 95$^0$C for 2 min, followed by 50 cycles<br>(95$^0$C for 15 s, 56$^0$C for 40 s), and 72$^0$C for 2 min |
| ***INHBC*** | forward<br>reverse | 5'- AgAgCTgCTTTgAggACTgC -3'<br>5'- AAgACgAgTCTggTTGATggTg -3' | 95$^0$C for 2 min, followed by 50 cycles<br>(95$^0$C for 15 s, 56$^0$C for 40 s), and 72$^0$C for 2 min |
| ***INHBE*** | forward<br>reverse | 5′- gCAACAATTCCTggCgATACC -3′<br>5′- gCCCTCAATTTCCCCTCCAC -3′ | 95$^0$C for 2 min, followed by 50 cycles<br>(95$^0$C for 15 s, 56$^0$C for 40 s), and 72$^0$C for 2 min |
| ***B2M*** | forward<br>reverse | commercially obtained by SABiosciences,<br>Cat No PPH01094E | 95$^0$C for 10 min, followed by 40 cycles<br>(95$^0$C for 15 s, 60$^0$C for 60 s) |

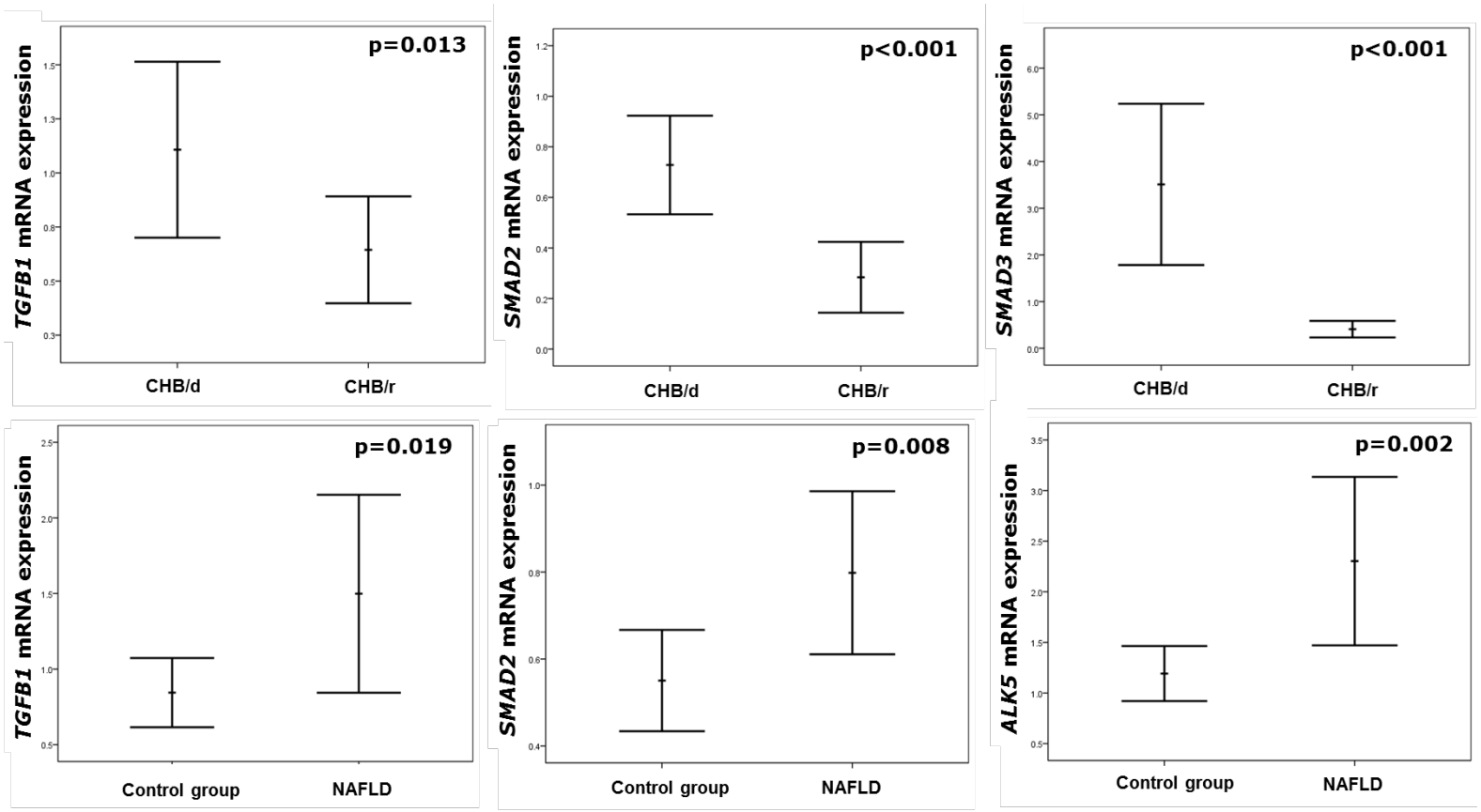
# Results(1)

- Patients with CHB/r exhibited a significant increase of *SMAD7* mRNA expression **(Fig.1A)** and reduced levels of *TGFB1*, *SMAD2*, *SMAD3*, and *CTGF* (p=0.010) as compared to CHB/d patients (**Fig.1B**). This pattern of expression of *SMAD7* was similar with that observed in patients with NAFLD, a disease characterized rarely by a fibrotic process **(Fig.1A)**.

# *SMAD7* mRNA expression



# TGFb1 mRNA expression



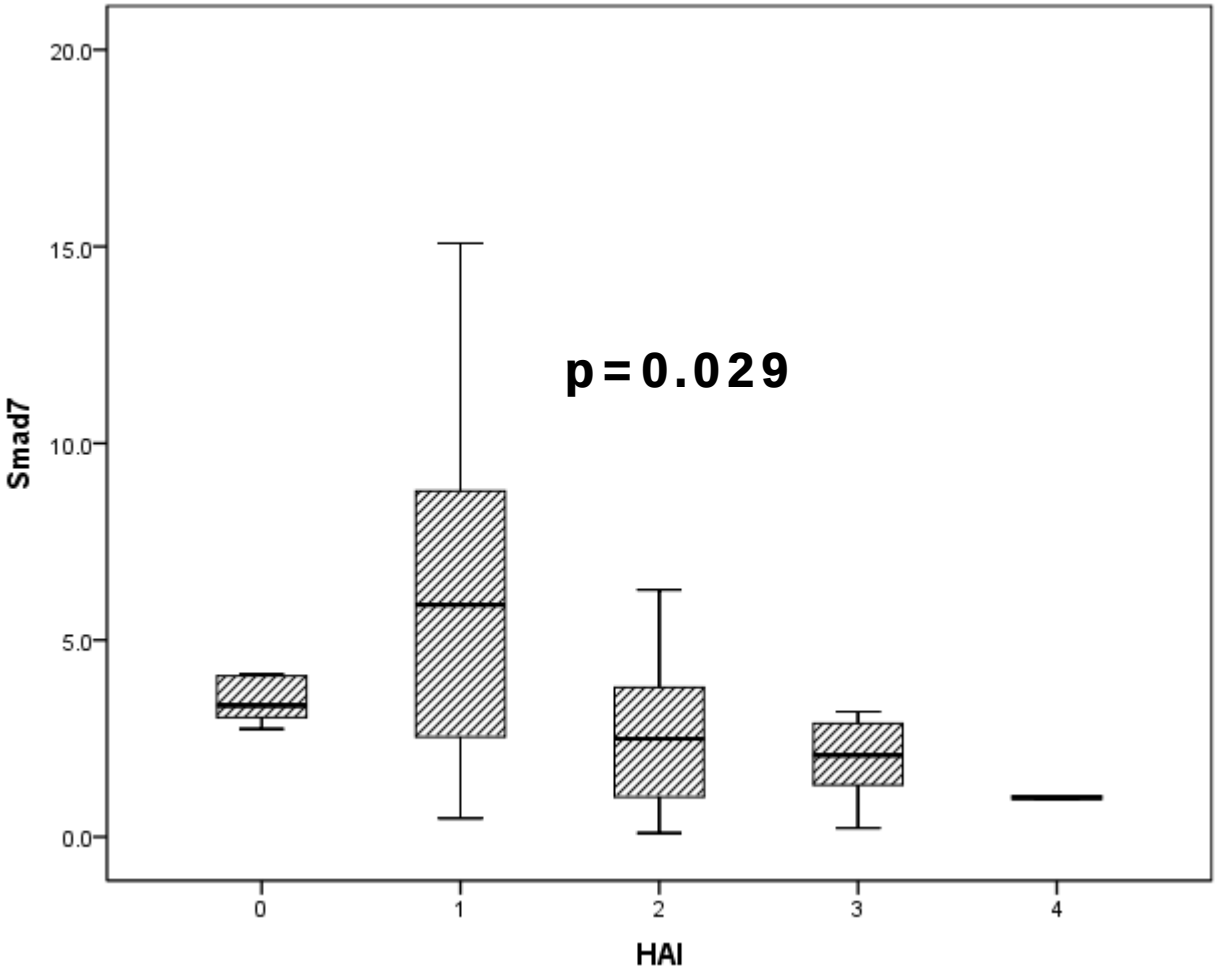
# Results(2)

- *SMAD7* expression was also found increased in NAFLD patients as compared to the control group including CHB/d, CHB/nr and CHC patients ($p=0.001$). Moreover, NAFLD patients were presented with elevated mRNA levels of *TGFB1*, *SMAD2*, *ALK4*, and *SMAD4* ($p<0.001$) (**Fig.1C**).

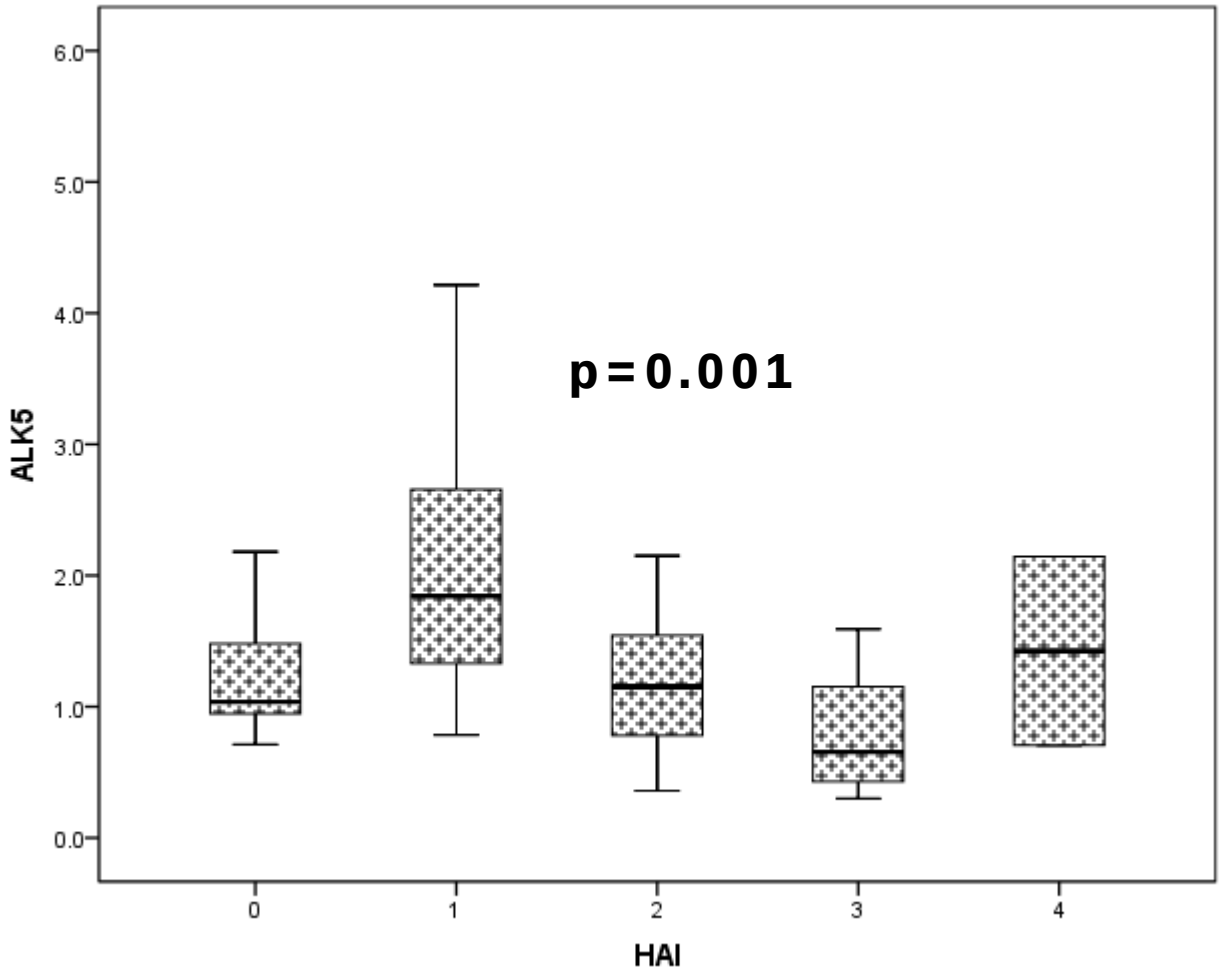
# Results(3)

- Considering the intensity of inflammation, *SMAD7*, *ALK5*, *INHBC*, and *ALK4* exhibited significant increased expression from absent to minimal inflammation with a gradual reduction as inflammation exacerbates (**Fig.2**).

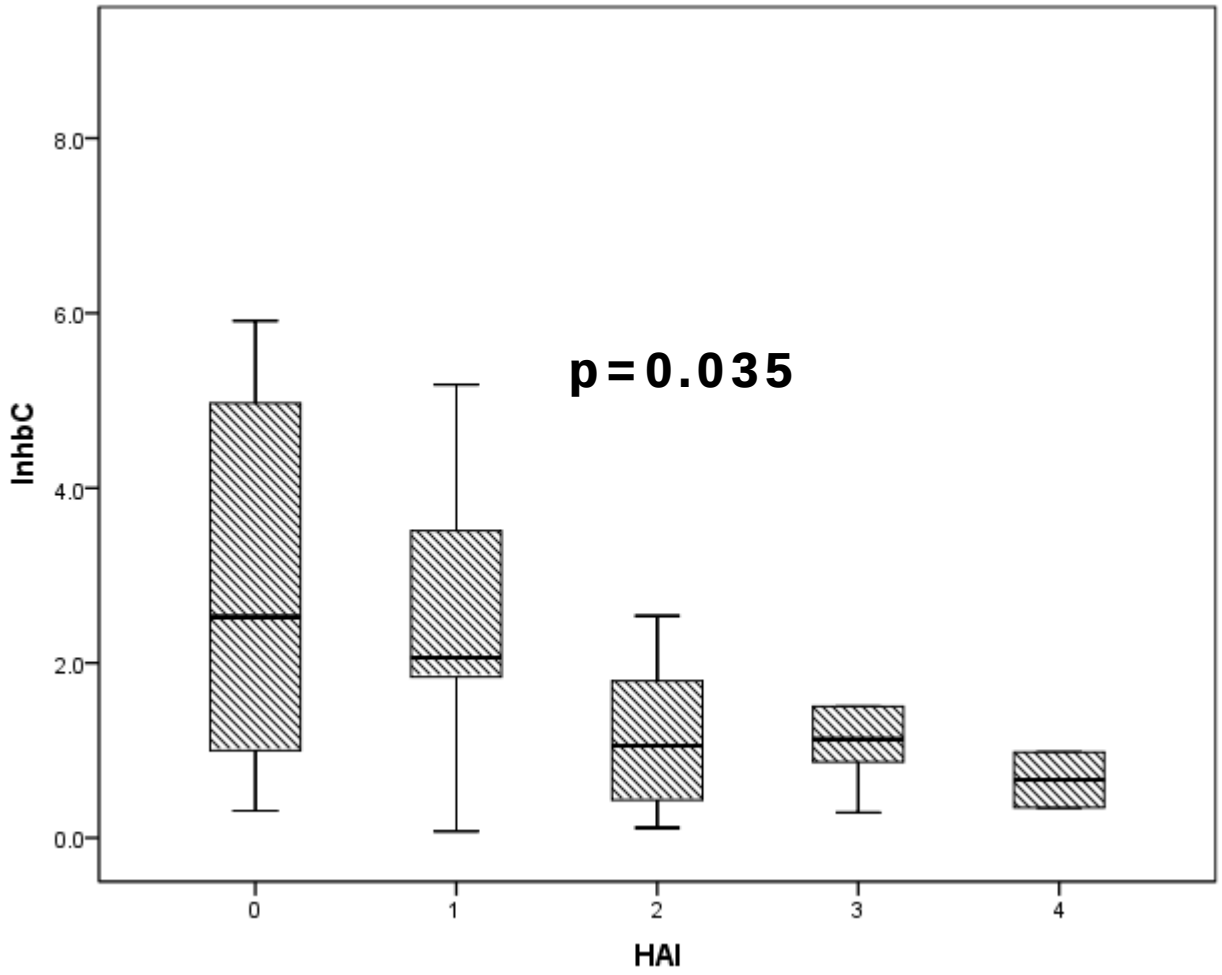
# *SMAD7*, *ALK5*, *INHBC*, and *ALK4* *vs* liver inflammation(TGF-b/ Activin signaling pathway )



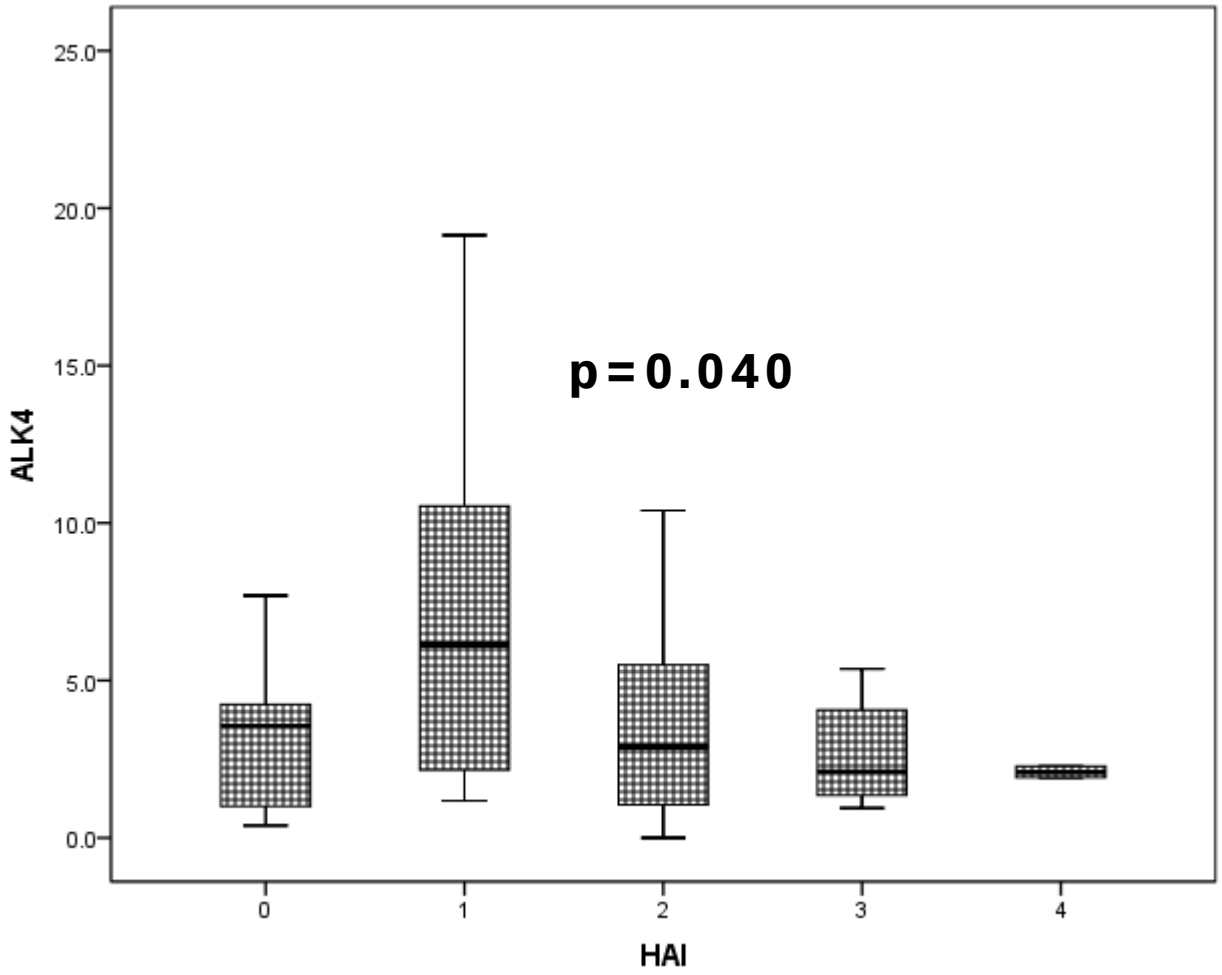
# *SMAD7*, *ALK5*, *INHBC*, and *ALK4* *vs* liver inflammation (TGF-b/ Activin signaling pathway )



# *SMAD7*, *ALK5*, *INHBC*, and *ALK4* vs liver inflammation (TGF-b/ Activin signaling pathway )



# *SMAD7*, *ALK5*, *INHBC*, and *ALK4* vs liver inflammation (TGF-b/ Activin signaling pathway )



# Conclusion

- Our data indicate that in cases with low grade fibrosis, such as
- NAFLD (characterized by a lower incidence of severe liver complications and fibrosis progression) and
- CHB/r, SMAD7 overexpression might be a mechanism limiting the fibrogenic effect of TGFb suggesting that its induction may provide a target for novel therapeutic approaches.

# REFERENCES


- **1.** Nakao A et al. Nature 1997; 389: 622-626
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- **4.** Dooley S et al. Gastroenterology 2008; 135: 642-659
- **5.** Tang LX et al. PLoS One 2012; 7: e31350. Epub 2012 Feb 7

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