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Supplemental Information

Aryl Hydrocarbon Receptor Promotes Liver Polyploidization and Inhibits PI3K, ERK, and Wnt/β-Catenin Signaling

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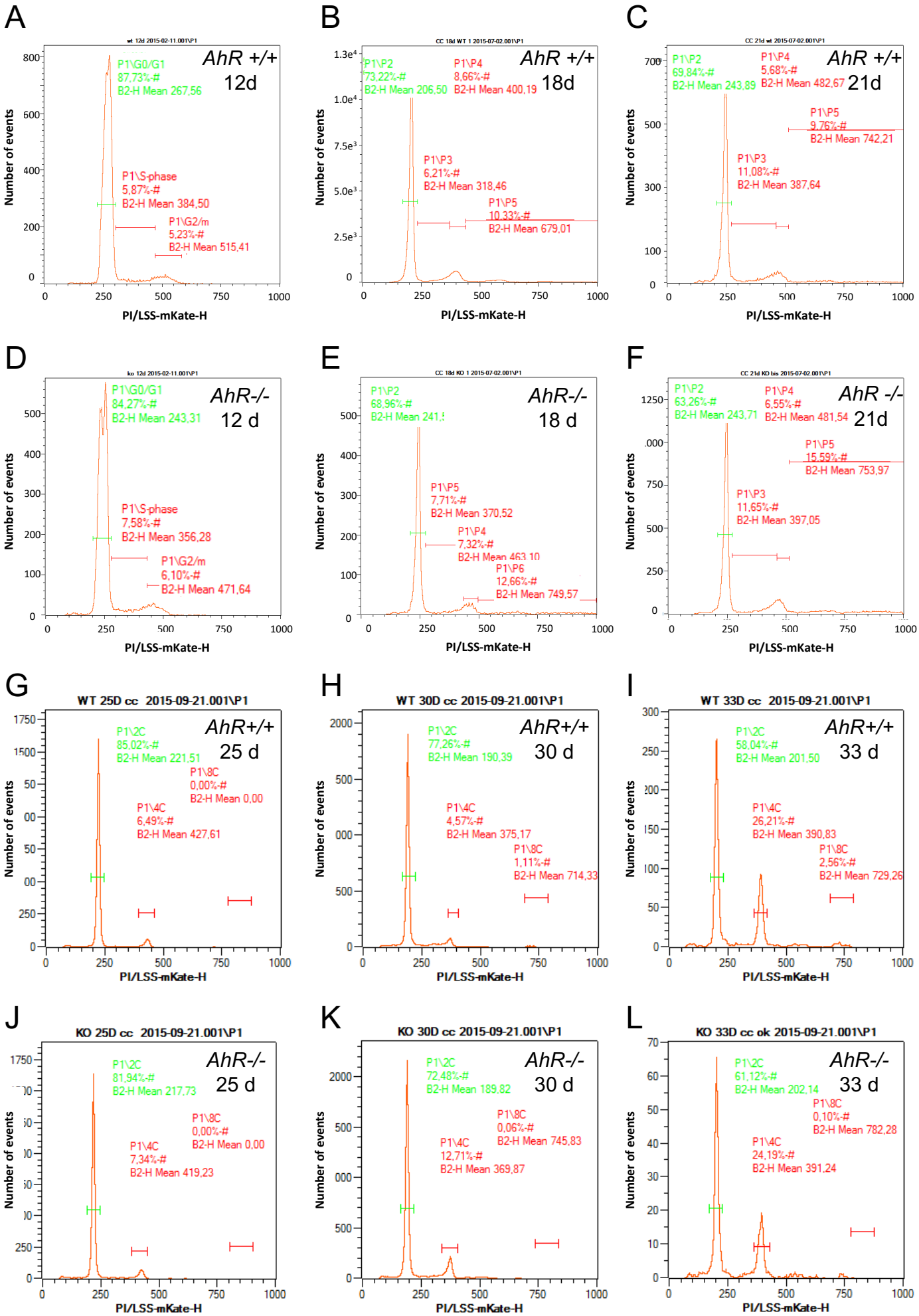
Supplementary Figure S1



LEGEND FOR SUPPLEMENTARY FIG. S1

Figure S1, related to Fig. 2. Ploidy analysis of preweaning mice. *AhR+/+* and *AhR-/-* preweaning mice between 12 and 33 days of age were analyzed for liver ploidy by flow cytometry. Primary hepatocytes were isolated at the indicated ages, processed for propidium iodide staining and their DNA content analyzed. Mice were at 12 days **(A,D)**, 18 days **(B,E)**, 21 days **(C,F)**, 25 days **(G,J)**, 30 days **(H,K)** and 33 days **(I,L)**. Peaks on the X-axis define DNA content and ploidy status.

Table S1, related to Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8 and Fig. 9

Antibodies used in this study

PROTEIN NAME	REFERENCE	BRAND
AhR	MA1-514	Thermo Scientific
INS-R	Ab131238	Abcam
p85-PI3K	06-497	Millipore-Upstate
PTEN	ab32199	Abcam
pAKT (Ser473)	5048	Cell Signaling
AKT	9272	Cell Signaling
p-GSK3β (Ser9)	5558P	Cell Signaling
GSK3β	12456P	Cell Signaling
pERK1/2 (Thr 202/Tyr204)	9101	Cell Signaling
ERK1/2	9102	Cell Signaling
p53	2524	Cell Signaling
$p21^{Waf1/Cip1}$	2947	Cell Signaling
$p27^{Kip1}$	3686	Cell Signaling
Cyclin E	sc-377100	Santa Cruz Biotechnology
mTOR	ab2732	Abcam
pP70 S6K1 (Tyr389)	9205	Cell Signaling
PCNA	307901	BioLegend
CES3	sc82554	Santa Cruz Biotechnology
Pericentrin	ab4448	Abcam
β-Actin	A2066	Sigma Aldrich
Histone H3	ab1791	Abcam
Cyclin B1	4138	Cell Signaling
Cyclin D1	2978	Cell Signaling
Albumin	4929	Cell Signaling

Table S2, related to Fig. 2, Fig. 6 and Fig. 9

Oligonucleotide primers used in this study

GENE NAME	DIRECTION	PRIMER SEQUENCE (5′-3′)
AhR	forward	AGCCGGTGCAGAAAACAGT
	reverse	AGGCGGTCTAACTCTGTGT
Cyp1a1	forward	ACAGACAGCCTCATTGAGCA
	reverse	GGCTCCACGAGATAGCAGTT
Axin 2	forward	ACTGGGTCGCTTCTCTTGAA
	reverse	CTCCCCACCTTGAATGAAGA
β-Cat	forward	CCCTGAGACCCTACATGAGG
	reverse	TGTCAGCTCAGGAATTGGAC
c-Myc	forward	CCTGACGACGAGACCTTCA
	reverse	TGGTAGGAGGCCAGCTTCT
Cyclin-D	forward	CACAACTTCTCGGCAGTCAA
	reverse	AGTGCGTGCAGAAGGAGATT
Dkk1	forward	GCAGGTGTGGAGCCTAGAAG
	reverse	GCCTCCGATCATCAGACTGT
Lef1	forward	GGGTGTTCTCTGGCCTTGT
	reverse	GCGACTTAGCCGACATCAA
Gapdh	forward	TGAAGCAGGCATCTGAGGG
	reverse	CGAAGGTGGAAGAGTGGGAG
Albumin	forward	TGCATCTAGTGACAAGGTTTGG
	reverse	GACTGGGGCCACTACTTCAA
mTOR	forward	CTCAAGCGATCCAGTTGTCA
	reverse	CAAAGAAGGGCTGAACTTGC

TRANSPARENT METHODS

Mice and treatments

Transgenic *AhR+/+* and *AhR-/-* mice were generated by gene targeting as previously described (Fernandez-Salguero et al., 1995). Preweaning male mice were used at around 25 days of age whereas adult mice were analyzed at 9-10 weeks of age. Previous studies have shown that weaning takes place in mice around 21 days and that polyploidization occurs between 3 and 4 weeks of age (Marques et al., 2008; Pandit et al., 2013; Pandit et al., 2012). Mice were sacrificed at the indicated times and liver tissue was removed and freshly processed for analysis or fixed for immunofluorescence. For metabolomic determinations, serum was obtained from clotted blood. Mice were also perfused through the portal vein of the liver to isolate primary hepatocytes as indicated below. All animal studies have been performed in accordance with the National and European legislation (Spanish Royal Decree RD53/2013 and EU Directive 86/609/CEE as modified by 2003/65/CE, respectively) and in accordance with the Institute of Laboratory Animal Resources (ILAR) for the protection of animals used for research. Experimental protocols were approved by the Bioethics Committee for Animal Experimentation of the University of Extremadura (Registry 109/2014), Junta de Extremadura (EXP-20160506-1) and National Cancer Institute Animal Care and Use Committee. Mice had free access to water and rodent chow.

Antibodies and reagents

The antibodies used in this study are indicated in Table S1. The AhR non-toxic ligand 6-formylindolo[3,2-b]carbazole (FICZ) was obtained from Thermo Scientific and it was used at 10 μM concentration. PI3K inhibitor LY294002, ERK inhibitor PD98059 and Wnt/β-cat inhibitor salynomicin were obtained from MedChem and were dissolved in sterile DMSO.

Reverse transcription and real-time PCR

Total RNA was purified from livers of preweaning and adult mice. Tissues were extracted in a Trizol reagent (Ambion)/chloroform solution, centrifuged and the supernatant precipitated with isopropanol. After centrifugation at 15000 *g* for 30 min at 4ºC, pellets were dissolved in DEPC-treated water and the crude RNA solution further purified using the High Pure RNA Isolation Kit (Roche). Reverse transcription was performed using random priming and iScript Reverse Transcription Super Mix (Bio-Rad). Real-time PCR (qPCR) was performed using SYBR® Select Master Mix (Life Technologies) in a Step One Thermal Cycler (Applied Biosystems) essentially as indicated (Morales-Hernandez et al., 2016; Rico-Leo et al., 2016). *Gapdh* was used to normalize target gene expression (ΔCt) and $2^{-\Delta\Delta Ct}$ to quantify changes in mRNA levels with respect to basal conditions. The oligonucleotide primer sequences used are indicated in Table S2.

SDS-PAGE electrophoresis and Western blotting

SDS-PAGE and Western blotting were performed using total protein liver extracts as described (Rico-Leo et al., 2016). In brief, *AhR*+/+ and *AhR*-/- liver tissues were homogenized in lysis buffer, centrifuged and protein concentration determined in the supernatants using the Coomassie Plus protein assay reagent (Pierce) and bovine serum albumin as standard. Aliquots of 20-30 μg total protein were electrophoresed in 8% SDS-PAGE gels which were transferred to nitrocellulose membranes by electroblotting. Following blocking in TBS-T solution containing 5% non-fat milk, membranes were sequentially incubated with the primary and the secondary antibodies, washed in TBS-T and revealed using the Super-signal luminol substrate (Pierce). Blots were scanned and protein expression quantified in a ChemiDoc XRS+ equipment (Bio-Rad).

Primary hepatocytes isolation by liver perfusion

To obtain primary hepatocyte cultures from mice, a two-step collagenase perfusion protocol was used essentially as described (Tanaka et al., 2015). Some technical modifications were introduced for *AhR-/-* mice in order to optimize perfusion efficiency due to their altered intrahepatic vasculature (Corchero et al., 2004; Lahvis et al., 2000). Mice were anesthetized, the internal organs set aside and the portal vein cannulated. The liver was initially rinsed with 40 ml of Hank's buffered salt solution (HBSS) containing 1 mM EDTA but without calcium and magnesium (Gibco) to remove blood. To drain the liver, the inferior vena cava was cut at the beginning of the process. Perfusion was done by passing 50 ml of a HBSS solution containing 0.6 mg/ml collagenases I and II (Gibco) and $CaCl_2$ through the cannulated portal vein. The liver was then removed, placed in a sterile tissue culture dish and the gallbladder carefully excised. Liver cells were gently detached, filtered through a 70 μm mesh cell strainer (Falcon) and centrifuged at 400 g for 3 minutes at 4°C. Primary hepatocytes were purified through a Percoll gradient (GE Healthcare), washed with PBS, stained with trypan blue to determine the number of dead cells and seeded in collagen-treated tissue culture plates at the appropriate cell density in DMEM-F12 medium (Lonza) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For some experiments, hepatocyte growth factor (HGF) and epidermal growth factor (EGF) were added to the medium.

***In vitro* and *in vivo* treatment with pharmacological inhibitors**

Primary hepatocyte cultures isolated from *AhR-/-* mice were seeded at a confluence of $1x10^6$ cells/well in 6-well plates and allowed to attach overnight. Cultures were then treated for 48 h with PI3K inhibitor LY294002 (25 and 50 μM), ERK inhibitor PD98059 (5 and 10 μM) and Wnt/β-cat inhibitor salynomicin (2,5 and 5 μM). Control cultures received the same concentration of the solvent DMSO. For *in vivo* inhibition of signaling

pathways, *AhR-/-* mice were treated for 7 days with a mix of 30 mg/kg LY294002 + 10 mg/kg PD98059 by i.p. injection plus 8 mg/kg salynomicin in drinking water. Control mice were injected with the same volume of DMSO. At the end of treatment, mice were sacrificed and their liver ploidy status analyzed by flow cytometry.

Ploidy analysis

Primary hepatocytes from *AhR+/+* and *AhR-/-* mice were fixed with 70% ethanol for 30 min at -20ºC and incubated with 10 μg/ml RNAse for 30 min at 37ºC. DNA content per cell was determined in a MACSQuant VYB (Miltenyi Biotech) after staining the cells with 50 μg/ml propidium iodide for 15 min at room temperature in the dark. Only signals from single cells were considered and at least 10.000 events were analyzed per sample. To quantify the number of binucleated cells, liver sections from preweaning and adult mice of each genotype were stained with DAPI and the number of hepatocytes containing two-nuclei counted using confocal fluorescence microscopy. Data are shown as the fraction of binucleated hepatocytes with respect to the total number of hepatocytes per field.

Wnt/β-Cat luciferase reporter assay

AhR+/+ and *AhR-/-* primary hepatocytes were seeded at a density of $4x10^5$ cells/well on Collagen-I pre-treated 12-well plates (Becton-Dickinson). After overnight incubation, cells were co-transfected with 1 μg TOPflash (TCF-binding sites-containing vector) or FOPflash (TCF-mutant binding sites-containing vector) expression plasmids (Millipore) and 0.4 μg pRL-TK (*Renilla* TK-luciferase vector, Promega) using Lipofectamine 3000 (Life Technologies). Luciferase activities were measured in a Varioskan Flash 96 Microplate Luminometer (Thermo Scientific) and, for each experimental condition, firefly luciferase (e.g. from TOPflash- or FOPflash-transfected primary hepatocytes) was normalized by *Renilla* luciferase. β-Cat-driven transcription was calculated as the

TOP/FOP ratio following normalization. In some experiments, primary hepatocytes were treated with Wnt3a-enriched medium (1:4 dilution) obtained from L1-Wnt3a cells grown in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Parallel cultures received medium from control L1 cells.

IRS-2 activation assay

Activation of the Insulin receptor signaling intermediate IRS-2 was determined by using the PathScan Phospho-IRS-2 ELISA Kit (Thermo Scientific). Briefly, sections of 50 mg liver tissue were homogenized with a MagNA Lyser equipment (Roche) in cell lysis buffer. Homogenates were then sonicated during 3 min on ice and protein concentration determined as indicated above. Aliquots of 100 μg and 250 μg total liver protein were added to the 96-well ELISA plate and the levels of pIRS-2 determined following the protocol provided by the manufacturer. Mice injected i.p. with 1 unit insulin were used as positive controls.

Immunoprecipitation

AhR+/+ and *AhR*-/- liver tissues were homogenized with a MagNA Lyser equipment (Roche) in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NaCl, 1% NP40, 10% glycerol, 2 mM EDTA, 1% PMSF and Halt protease inhibitor cocktail (Thermo Scientific)). Homogenates were centrifuged at 10000 g for 5 min at 4°C and protein concentration was quantified using the BCA protein assay kit (Beyotime Biotech). Aliquots of 2 mg liver protein were immunoprecipitated using Dynabeads Protein G (Life Technologies) and antibodies against AhR and pAKT essentially as described (Rey-Barroso et al., 2014; Rico-Leo et al., 2013).

Hematoxylin/eosin staining of liver sections

Livers from *AhR*+/+ and *AhR*-/- mice were fixed overnight at room temperature in buffered formalin and included in paraffin. Sections of 3 μm were deparaffinated in xylol

and re-hydrated to phosphate buffered saline (PBS). Sections were incubated for 3 min with hematoxylin, washed with tap water and stained with eosin for 1 min. Sections were de-hydrated, mounted and observed in a NIKON TE2000U microscope using 4x (0.10 numeric aperture) and 10x (0.25 numeric aperture) objectives.

Immunofluorescence and confocal microscopy

Liver sections prepared as indicated above were incubated for 1 h at room temperature in PBS containing 0.25% Triton X-100 (PBS-T), 0.2% gelatin and 3% BSA to block unspecific epitopes. Sections were incubated overnight at 4ºC with anti-β-Cat primary antibody diluted in PBS-T containing 0.2% gelatin. After washing in the same solution, tissues were incubated for 1 h at room temperature with an Alexa-633-labeled secondary antibody. After further washing, sections were dehydrated, mounted on Mowiol and visualized using an Olympus FV1000 confocal microscope (Olympus). For some experiments, 10 μm liver sections were processed as indicated above and incubated with anti-PCNA primary antibody and Alexa-488 secondary antibody. Sections were analyzed with the same confocal microscope equipped with a multiphoton laser. Objectives used were: 10x (0.40 numeric aperture) and 20x (0.70 numeric aperture). Fluorescence analysis was done using the FV10 software (Olympus). DAPI was used to stain cell nuclei.

Metabolomic analyses by GC-MS

Blood was collected from *AhR*+/+ and *AhR*-/- group mice and serum prepared using Serum Separator Tubes (Becton-Dickinson) as indicated by the manufacturer. GC-MS analysis was performed with an Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass-selective detector (MSD) as previously described (Patel et al., 2017) for the tricarboxylic cycle intermediates, amino acid (L-Leucine, L-Glutamine) and dicarboxylic acid (Azelaic acid) analysis. Additionally, m/z (Qualifier ions 1 -Q1, Qualifier ions 2 -

Q2) for L-leuicine 200 (274,302), and Azelaic acid 317 (359,201) at retention time 13.3 min and 18.8 min in sequence were selected for single ion monitoring by GC-MS. Data Processing were conducted with agilent mass hunter work station software.

Metabolomics profiling with UPLC-ESI-QTOFMS

UPLC-ESI-QTOFMS analysis was performed as previously reported (Matsubara et al., 2012). Samples were introduced into the mass spectrometer Q-TOF Premier (Waters Corporation) operating in either negative or positive electrospray ionization modes. Data processing and multivariate data analysis were conducted as previously reported (Matsubara et al., 2012). A PCA, PLS-DA and contribution analyses were performed using SIMCA-P+12 (Umetrics).

Statistical analyses

Quantitative data are shown as mean ± SD. Comparisons between experimental conditions was done using GraphPad Prism 6.0 software (GraphPad). The student´s t test was used to analyze differences between two experimental groups and ANOVA for the analyses of three or more groups. The Mann-Whitney non-parametric statistical method was used to compare rank variations between independent groups.

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