Supplemental methods

The ODYSSEY CHOICE II study

We assessed the effect of 24-weeks of treatment with Alirocumab in a single-center substudy of the ODYSSEY CHOICE II (NCT02023879). The ODYSSEY CHOICE II was a randomized, double-blind, placebo-controlled, Phase 3 multinational study including 233 patients from 43 study sites. The CHOICE II study enrolled adult patients with hypercholesterolemia, (1) not receiving a statin due to statin associated muscle symptoms (SAMS) with moderate, high, or very-high cardiovascular risk, or (2) not receiving a statin but who did not fulfill the SAMS definition. CHOICE II comprised 24 weeks of double-blind treatment with Alirocumab 150 mg every 4 weeks (Q4W), Alirocumab 75 mg every 2 weeks (Q2W), or placebo Q2W, respectively. Patients were randomized as follows to one of the three arms, placebo for Alirocumab, Alirocumab 75 Q2W/up 150 Q2W, or Alirocumab 150 Q4W/Up 150 Q2W in a planned 1:1:2 ratio, during the double-blind treatment period:

- Placebo for Alirocumab subcutaneous (SC) Q2W starting at Week 0 (randomization) and continuing up to Week 22, i.e. 2 weeks before the end of the double-blind treatment period.

- Alirocumab SC 75 mg Q2W starting at Week 0 (randomization) up to Week 12. Based on the patients' Week 8 LDL-C level, patients either continued with Alirocumab 75 mg Q2W or had their dose up-titrated to 150 mg Q2W up to Week 22, i.e. 2 weeks before the end of the double-period.

- Alirocumab SC 150 mg Q4W starting at Week 0 (randomization) up to Week 12. Based on the patients' Week 8 LDL-C level, patients either continued with Alirocumab 150 mg Q4W or had their dose up-titrated to 150 mg Q2W up to Week 22, i.e. 2 weeks before the end of the double-period (the blind was maintained in patients receiving Alirocumab 150 Q4W by alternating with placebo SC Q4W).

After study completion, all patients were offered the opportunity to enter an open-label extension study during which all patients received Alirocumab 150 mg Q4W.

Migratory capacity: Trans-endothelial migration (TEM) and chemotaxis

Primary human arterial endothelial cells (Lonza, Baltimore, MD) were cultured to confluence. After overnight stimulation with 10 ng/mL tumor necrosis factor- α (TNF-α, Peprotech, London, United Kingdom), CD14+ MACS-sorted (Milteny Biotec, Leiden, The Netherlands) isolated monocytes susepended in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich, Zwijndrecht, The Netherlands) were added at a concentration of $1*10^6$ cells/mL for 30 min at 37°C, 5% CO2 and then fixed with 3.7% formaldehyde (Sigma-Aldrich, Zwijndrecht, The Netherlands). Images were recorded with a Zeiss Axiovert 200 microscope (Plan-apochromat 10x/0.45 M27 Zeiss-objective; Carl Zeiss Inc., Jena, Germany). Transmigrated monocytes were distinguished from adhered monocytes by their transitions from bright to dark morphology. Quantification was performed by using the cell counter plugin (http://rsbweb.nih.gov/ij/plugins/cell-counter.html) in the Image-J software (http://rsb.info.nih.gov/nih-image/). To investigate whether migration was mediated by MCP-1 a chemotaxis assay was performed**.** The EZ-TAXIScan chamber (Effector Cell Institute, Tokyo, Japan) was assembled with a 260 µm wide and 4 µm thick silicon chip coated with Fibronectin (FN) (30 µg/ml) for 1 h at 37°C and subsequently blocked with 2% Bovine Serum Albumin (BSA) phosphate buffered saline (PBS) for 30 min. The chamber was assembled on a 30 mm round FN-coated (30 µg/mL) glass coverslip and filled with monocyte migration medium (RPMI/0.1% BSA). Isolated monocytes ($1*10^6$ cells/mL) were added to the upper reservoir of each of the six channels and allowed to line up by removing 10 µL from the lower reservoir. Five µL monocyte chemoattractant protein-1 (100 ng/mL) was added to the lower reservoir and monocyte migration was captured every 120 s for 120 min using a 10x objective. Cell migration was quantified using ImageJ and the available manual tracking plugin (http://rsb.info.nih.gov/ij/plugins/manual-tracking.html). This plugin allowed us to manually track monocyte migration. Obtained datasets were further analyzed using the chemotaxis tool

plugin (version 1.1) from Ibidi (Ibidi, Planegg, Germany). At least 30 cells per condition were tracked. Distance towards MCP-1 (either positive or negative) was averaged to calculate mean migration distance towards MCP-1.

Monocyte preparation for lipid accumulation assessment

Monocytes suspended in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich, Zwijndrecht, The Netherlands) were added to fibronectin (FN; 30 µg/mL) coated glass slides ($0.5*10^6$/mL), incubated for 1 hour (37°C, 5% CO_2), fixed with 4% formaldehyde , permeabilized with 0.1% Triton-X100 and incubated with the lipid dye Nile Red (1 µg/mL; N3013-100MG, Sigma Aldrich, Zwijndrecht, The Netherlands), after which cells were mounted (Dako, Heverlee, Belgium).

FACS

Freshly isolated monocytes were stained for chemokine receptor (CCR) 2 (Novus Biologicals, Littleton, CO) expression and subsequently cells were sorted by fluorescent activated cell sorting (FACS). Cells with a median fluorescence intensity higher than 3500 were quantified as CCR2 high expressing cells, whereas cells with a median MFI lower than 3500 were quantified as CCR2 low and intermediate-expressing cells. Next, sorted cells were spread on a FN-coated microscopy slide and stained for Nile red as described.

Co-immuno staining

For the co-immuno staining of CCR2 and Nile Red, after fixation cells were blocked for 15 minutes with 2% BSA/PBS. Subsequently cells were stained with CCR2 Alexa 405 (Novus Biologicals, Littleton, CO) in 1% BSA/PBS for 45 minutes, washed with PBS and incubated with the lipid dye Nile red as described.

***Ex vivo* lipopolysaccharide (LPS) challenge for cytokine production**

$0.2*10^6$ cells were plated in a 96-well tissue culture plate for 45 min allowing monocyte adherence in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich, Zwijndrecht, The

Netherlands) supplemented with 2 mM l-glutamine, penicillin (100 U/mL), streptomycin (100 µg/ml) and 1% fetal calf serum (FCS; all Gibco, Waltham, MA) $0.2*10^6$ cells were plated in a 96-well tissue culture plate for 45 min allowing monocyte adherence in Iscove's Modified Dulbecco's Medium (IMDM, Sigma-Aldrich, Zwijndrecht, The Netherlands) supplemented with 2 mM l-glutamine, penicillin (100 U/mL), streptomycin (100 µg/ml) and 1% fetal calf serum (FCS; all Gibco, Waltham, MA). Hereafter, the medium was removed and replaced by IMDM with 10% FCS.

Supplemental Table S1: Markers used for flow cytometry

Surface marker	Color	Dilution	Company
CD14	PE-Cy7	1/50	BD
CD16	APC-Cy7	1/50	BD
HLA-DR	PerCpCy5.5	1/50	BD
CCR2	APC	1/50	BD
CCR5	FITC	1/10	BD
CX3CR1	PE	1/50	Biolegend
CD18	APC	1/50	BD
CD29	APC	1/10	BD
CD11b	PE	1/25	BD
LDLR	PE	1/25	BD
SR-A	PE	1/10	R&D
CD36	APC	1/25	BD
Mouse IgG1 κ isotype control	FITC	1/50	BD
Mouse IgG1 κ isotype control	PE	1/50	BD
Mouse IgG1 κ isotype control	APC	1/50	BD

APC, allophycocyanin; CD, cluster of differentiation; CR, chemokine receptor; Cy, CyChrome; FITC, Fluorescein isothiocyanate; HLA, human leucocyte antigen; LDLR, Low density lipoprotein receptor; PE, phycoerythrin ; PerCP, peridinin-chlorophyll-protein; SR, scavenger receptor.

Supplemental Table S2: Primer sequences for qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ABCA1	CCTGTCATCTACTGGCTCTC	ACAACGTAATTGCACATATCCC
ABCG1	GAGATGGGAGTCTTTCTTCGG	CACTGGGAACATGATCTGAAAGG
B2M	AGCGTACTCCAAAGATTCAGGTT	ATGATGCTGCTTACATGTCTCGAT
GAPDH	GAAGGTGAAGGTCGGAGTCAAC	CAGAGTTAAAAGCAGCCCTGGT

ABCA, ATP binding cassette transporter; B2M, beta-2-microglubilin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Supplemental Table S3: Disease specifications of the Familial Hypercholesterolemia patients not receiving statin therapy. Corresponding to Group 1: Adult subjects with definite or probable FH, based on the DLN criteria currently untreated

Characteristic	FH patients, no statin (n=22)
Diagnosis	
- FH, genetic mutation, n (%)	13 (59)
- FH, clinical diagnosis (DLN criteria), n (%)	9 (41)
Medication use	
- Ezetrol, n (%)	15 (68)
- Antihypertensive, n (%)	6 (27)

Data are n (%).

FH, familial hypercholesterolemia. DLN; Dutch Lipid Network.

Supplemental Table S4: Clinical characteristics of the Familial Hypercholesterolemia cohort receiving Evolocumab and controls: both included for flow cytometry, migratory and immunohistochemical experiments

Values are n (%), mean ± SD or median [IQR,] for skewed data.

	Control n=7	**FH pre-Evolocumab n=7**	***P*-value**	***FH* post-Evolocumab**	***P*-value**
Age, years	56±8	61±11	0.338	n/a	n/a
Gender, n, male (%)	3(43)	3(43)	1.000	n/a	n/a
BMI, kg/m²	24±3	27±5	0.200	27±5	0.302
Smoking, (% active)	0(0)	0(0)	1.000	n/a	n/a
CVD history	0(%)	3(43)	0.051	n/a	n/a
SBP, mmHg	125±14	125±15	0.953	135±16	0.221
DBP, mmHg	77±10	82±9	0.448	81±5	0.783
CRP, mg/L	0.6 [0.2–1.9]	2.9 [0.4–4.2]	0.181	1.2[0.8-2.6]	0.735
Total cholesterol, mmol/L	5.3±1.0	9.8±1.9	<0.001	6.1±1.6	0.001
LDL-C, mmol/L	3.0±0.5	7.8±1.9	<0.001	4.0±1.3	<0.001
HDL-C, mmol/L	1.8±0.7	1.3±0.5	0.143	1.5±0.6	0.01
Triglycerides, mmol/L	0.8 [0.2–1.9]	1.49 [1.1–1.7]	0.432	[0.7-1.5]	0.043
Leucocytes, 10^9/L	5.1±0.9	6.3±2.4	0.265	6.0±2.0	0.498
Neutrophils, 10^9/L	3.0±0.7	3.7±1.4	0.284	3.5±1.3	0.541
Lymphocytes, 10^9/L	1.5±0.3	1.9±1.0	0.278	1.8±0.5	0.665
Monocytes, 10^9/L	0.4±0.1	0.5±0.2	0.407	0.5±0.2	0.430

BMI, body mass index; CRP; c-reactive protein; CVD, cardiovascular disease; DBP; diastolic blood pressure; FH, familial hypercholesterolemia; HDL-C; high density lipoprotein cholesterol; IQR, inter-quartile range; LDL-C; low density lipoprotein cholesterol; Lp(a); lipoprotein (a); SBP; systolic blood pressure, SD, standard deviation.

Supplemental Table S5 Treatment allocation of Alirocumab treated patients

Subject	Double-blind period	Uptitrated at Week 12 in the DB	OLE	Sample used
1	75 MG Q2W	Yes 150 mg Q2W	150 mg Q4W	Week 24 double-blind period With 150Q2W
2	75 MG Q2W	Yes 150 mg Q2W	150 mg Q4W	Week 24 double-blind period With 150Q2W
3	75 MG Q2W	Yes 150 mg Q2W	150 mg Q4W	Week 24 double-blind period With 150Q2W
4	150 MG Q4W	Yes 150 mg Q2W	150 mg Q4W	Week 24 double-blind period With 150Q2W
5	150 MG Q4W	No 150 mg Q4W	150 mg Q4W	Week 24 double-blind period With 150Q4W
6	Placebo	n/a	150 mg Q4W Up-titrated in the OLE	Week 24 OLE With 150Q2W
7	150 MG Q4W	Yes 150 mg Q2W	150 mg Q4W	Week 24 double-blind period With 150Q2W
8	Placebo	n/a	150 mg Q4W Up-titrated in the OLE	Week 24 OLE With 150Q2W
9	Placebo	n/a	150 mg Q4W Up-titrated in the OLE	Week 24 OLE With 150Q2W
10	75 MG Q2W	Yes 150 mg Q2W	150 mg Q4W	Week 24 double-blind period With 150Q2W

150Q2W =150 mg every 2 weeks; 150Q4W, 150 mg every 4 weeks; 75Q2W, 75 mg every 2 weeks; LMT, lipid-modifying treatment; n/a, not applicable; OLE, open label extension.

Supplemental Table S6 Clinical characteristics of Familial Hypercholesterolemia patients (no statin) pre- and post Alirocumab treatment

	FH, pre Alirocumab *n=10*	**FH, post Alirocumab** *n=10*	***P*-value**
Age, years	54±12	54±12	n/a
Gender, n, male (%)	6 (60)	6 (60)	n/a
BMI, kg/m^2	28±5	29±5	0.342
Smoking (% active)	1 (10)	1 (10)	n/a
CVD history (%)	6 (60)	6 (60)	n/a
SBP, mmHg	137±11	133±19	0.488
DBP, mmHg	82±10	79±9	0.407
CRP, mg/L	1.2 [0.7-2.4]	1.2 [0.6-1.7]	0.600
Total cholesterol, mmol/L	8.1±1.2	5.1±1.4	<0.001
LDL-C, mmol/L	5.8±1.3	2.9±1.2	<0.001
HDL-C, mmol/L	1.5±0.6	1.4±0.5	0.645
Triglycerides, mmol/L	1.7 [1.3–2.7]	1.5 [0.9–2.4]	0.069
Leukocytes, 10^9/L	6.0±0.8	6.0±0.5	0.864
Neutrofils, 10^9/L	3.7±0.6	3.7±0.8	0.869
Lymphocytes, 10^9/L	1.7±0.3	1.7±0.4	0.949
Monocytes, 10^9/L	0.4±0.1	0.5±0.1	0.673

Values are n (%), mean±SD or median [IQR,] for skewed data.

BMI, body mass index; CRP; c-reactive protein; CVD, cardiovascular disease; DBP; diastolic blood pressure; FH, familial hypercholesterolemia; HDL-C; high density lipoprotein cholesterol; IQR, inter-quartile range; LDL-C; low density lipoprotein cholesterol; n/a, not applicable; SBP; systolic blood pressure, SD, standard deviation.

Supplemental Table S7: Disease specifications of the Familial Hypercholesterolemia patients (no statin) receiving PCSK9 monoclonal antibodies and FH patients on stable statin therapy

Characteristic	FH, Alirocumab (n=10)	FH, Evolocumab (n=7)	FH, Stable statin (n=14)
Diagnosis			
- FH, genetic mutation	5 (50)	2 (29)	7 (50)
- FH, clinical diagnosis (DLN criteria)	5 (50)	5 (71)	7 (50)
Medication use			
- Ezetrol, n (%)	9 (90)	4 (60)	6 (43)
- Antihypertensive, n (%)	5 (50)	0(0)	2 (17)
- statin	0	0	14

Data are n (%).

FH, familial hypercholesterolemia; DLN; Dutch Lipid Network; PCSK9, proprotein convertase subtilisin/kexin type 9.

Supplemental Figure legends

Supplemental Figure legends

Supplemental Figure S1. Expression of chemokine receptors and integrins in Familial Hypercholesterolemia patients

Flow cytometry on whole blood was performed to study monocyte surface expression of chemokine receptors and integrins in FH-patients (n=22, filled squares) versus controls (n=18, open circles). Surface expression of CX3CR1 **(A)**, CCR5 **(B)**, CD11b **(C)**, CD18 **(D),** and CD29 **(E)** is represented as delta median fluorescence intensity. **(F)** Histogram of LDLR surface expression as assessed by flow cytometry in 3 individual donors. Dashed lines indicate isotype controls and black solid lines the LDLR . Data represent mean ± SD **P*<0.05; ***P*<0.01; ****P*<0.001. P values are unadjusted.

CD, cluster of differentiation; CCR, c-motif chemokine receptor; FH, familial hypercholesterolemia; LDLR, low density lipoprotein receptor; SEM, standard error of the mean.

Supplemental Figure S2. Co-occurrence of CCR2 expression and lipid accumulation in isolated monocytes. (A) shows a representative HPLC chromatogram of a lipid droplets extracted from three different samples and a blanco. CE and TAG peaks are indicated by black arrows. **(B)** Normalized peak area of CE content of lipid droplets**. (C,D)** Monocytes were separated with FACS, distinguishing high (MFI >3500) and intermediate or low (MFI <3500) CCR2 expression. Lipid content of CCR2 low/intermediate and high subsets assessed by NR immunohistochemistry. **(E)** Co-immunohistochemistry of CCR2 (purple) and neutral lipids (green). Data represent mean ± SD. ****P*<0.001. P values are unadjusted.

CCR, c-motif chemokine receptor; FACS, fluorescence activated cell sorting; MFI, median fluorescence intensity; NR, Nile Red.

Supplemental Figure S3. Expression of chemokine receptors and integrins after treatment

Flow cytometry on whole blood was performed to study monocyte surface expression of

chemokine receptors and integrins after lipid lowering. FH, baseline (n=17, filled squares), FH, post PCSK9 mAbs (n=17, open squares), FH, stable statin (n=14, open triangles). **(A)** Percentage of monocyte subsets, divided into classical, (CD14++/CD16-), intermediate (CD14++/CD16+), or non-classical (CD14dim/CD16+). Surface expression of CX3CR1 **(B)**, CCR5 **(C)**, CD11b **(D)**, CD18 **(E),** and CD29 **(F)** is represented as delta median fluorescence intensity. Data represent mean ± SD. **P*<0.05; ***P*<0.01; ****P*<0.001. P values are unadjusted.

CD, cluster of differentiation; CCR, c-motif chemokine receptor; FH, familial hypercholesterolemia; PCSK9, proprotein convertase subtilisin/kexin type 9; SEM, standard error of the mean.

Supplemental figure S4. Monocytes of Familial Hypercholesterolemia patients show increased migration towards MCP-1. Representative image **(A)** and plots **(B)** of migration towards a MCP-1 gradient, observed in a control subject **(left)**, FH (no statin) **(middle)**, and FH PCSK9 mAb treated **(right).** Migration is quantified in 30 cells per subject as migrated distance in μm, distance in red (towards MCP-1) is considered positive, distance in blue (away from MPC-1) is considered negative, all values are used to calculate average migration towards MCP-1 **(C).** Data represent mean ± SD.

FH, familial hypercholesterolemia; MCP-1, monocyte chemoattractant protein-1; PCSK9, proprotein convertase subtilisin/kexin type 9.

Supplemental figure S5. CCR2 expression and lipid accumulation for Evolocumab and Alirocumab. To assess the effect of PCKS9 mABs by different antibodies, FH patients were treated for 6 months with Evolocumab or Alirocumab. FH, pre- (n=7, filled diamond) and post-Evolocumab (n=7, open diamond), FH pre- (n=10, filled hexagon) and post-Alirocumab (n=10, open hexagon). (A) Surface expression of monocyte CCR2 represented as delta median fluorescence intensity. (B) Quantification of intracellular lipid accumulation using NR immunohistochemistry presented as the percentage of lipid positive cells. (C) Number of lipid

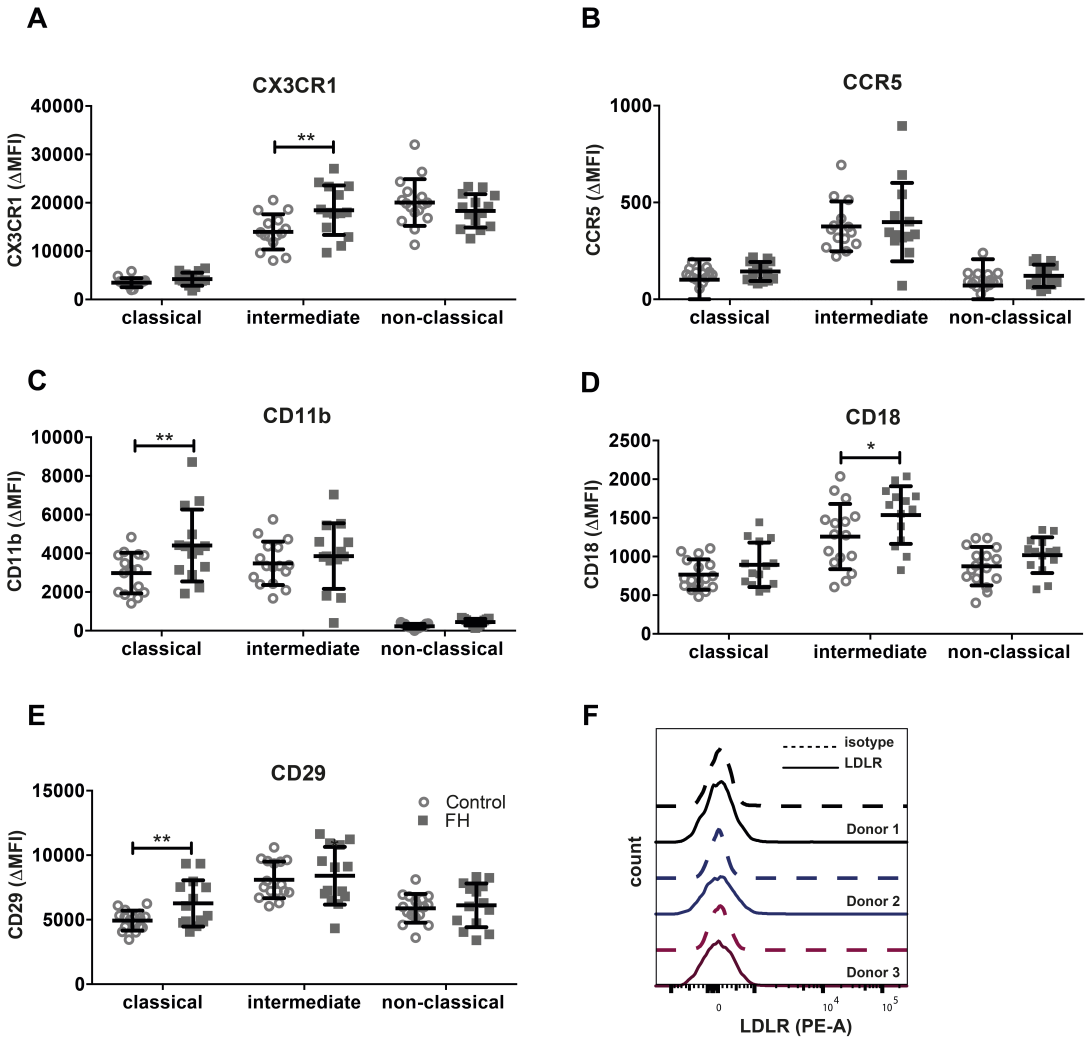
droplets per lipid positive cell. Data represent mean ± SD *P<0.05; **P<0.01; ***P<0.001. P values are unadjusted.

CCR, c-motif chemokine receptor; FH, familial hypercholesterolemia; NR, Nile Red; PCSK9, proprotein convertase subtilisin/kexin type 9.

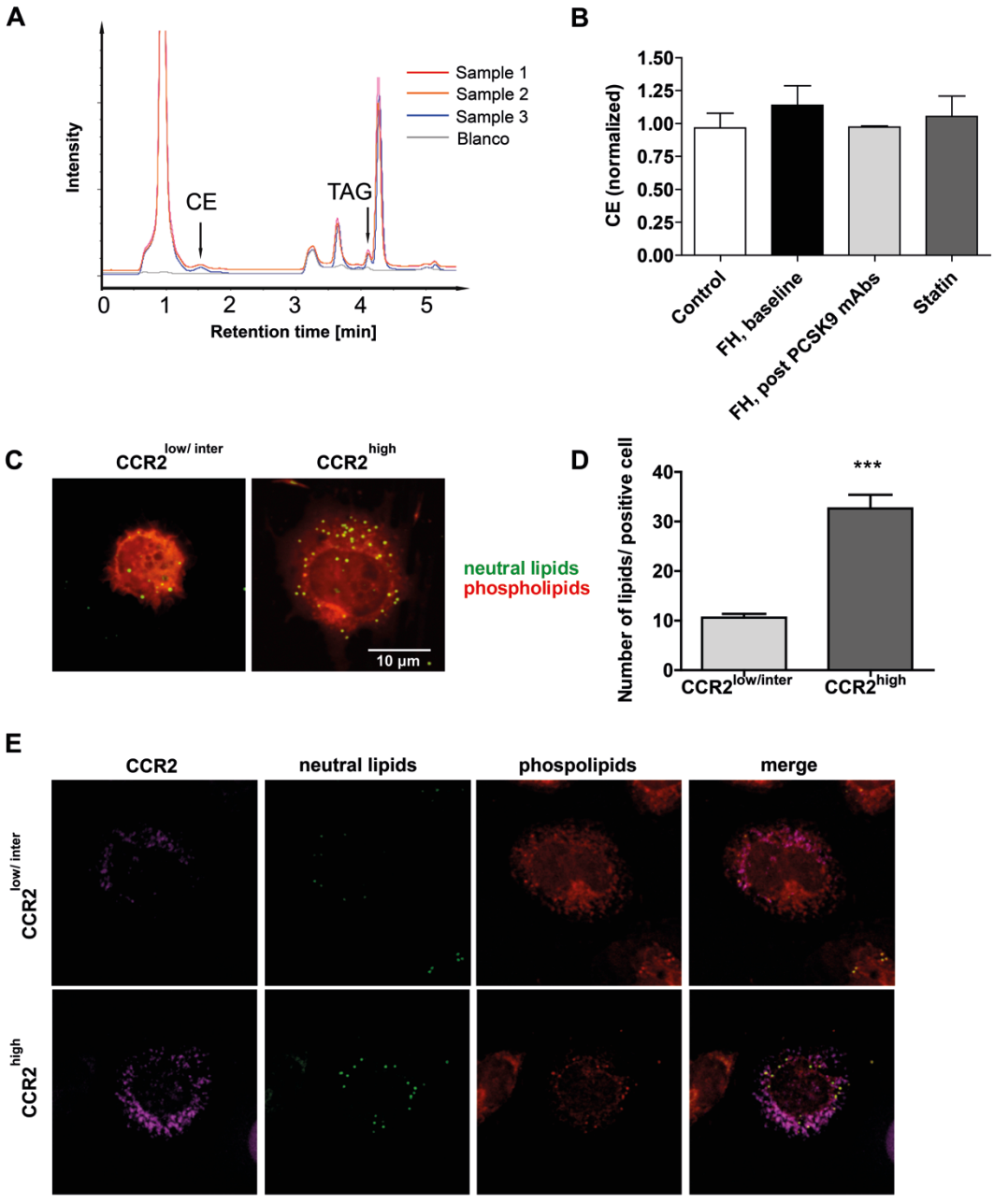
Supplemental Video. Monocytes of Familial Hypercholesterolemia patients show increased migration towards MCP-1. Video of representative experiment performing a MCP-1 chemotaxis assay. Over a 2 h period, monocytes of an FH patient (**middle**, LDL: 7.62 mmol/L) move rapidly towards an MCP-1 gradient, whereas monocytes of a control subject (**left**: LDL 2.53 mmol/L) do not show significant directional movement. After PCSK9 mAb treatment (**right**: LDL 3.03 mmol/L) monocyte movement is slower compared to the FH patient (no statin).

FH, familial hypercholesterolemia; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; PCSK9, proprotein convertase subtilisin/kexin type 9.

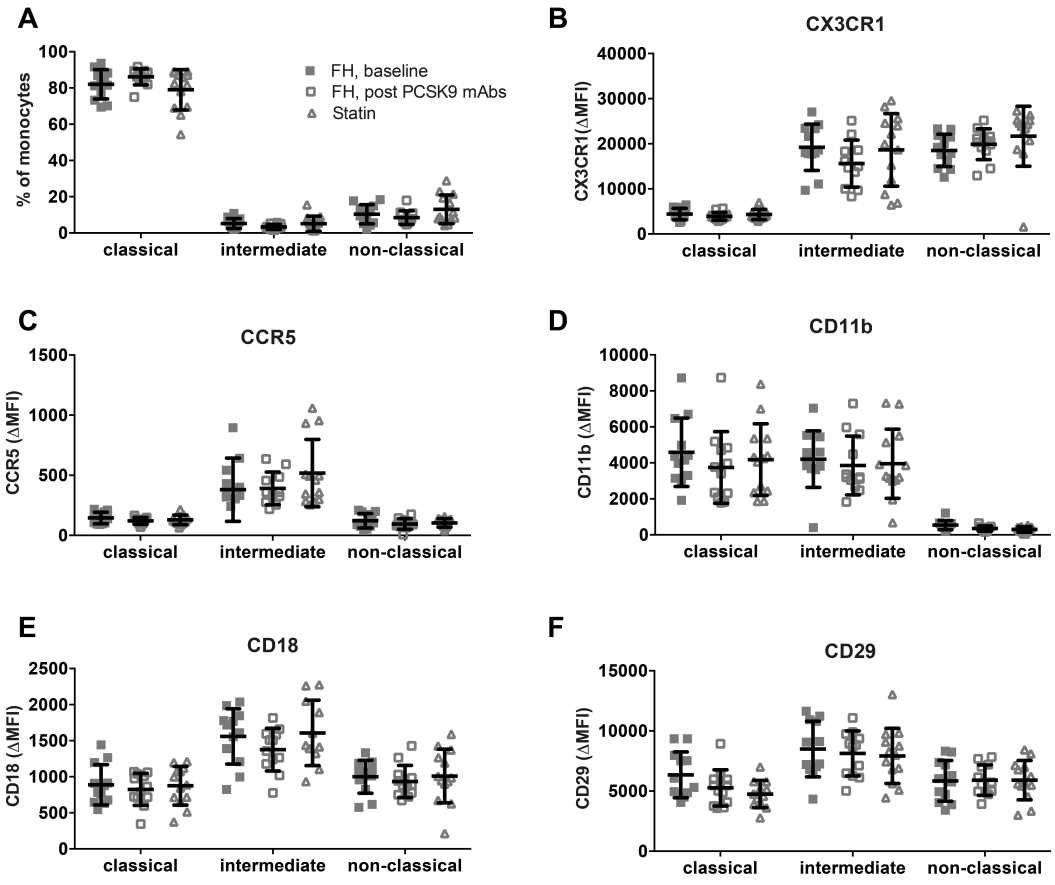
Supplemental Figure 1



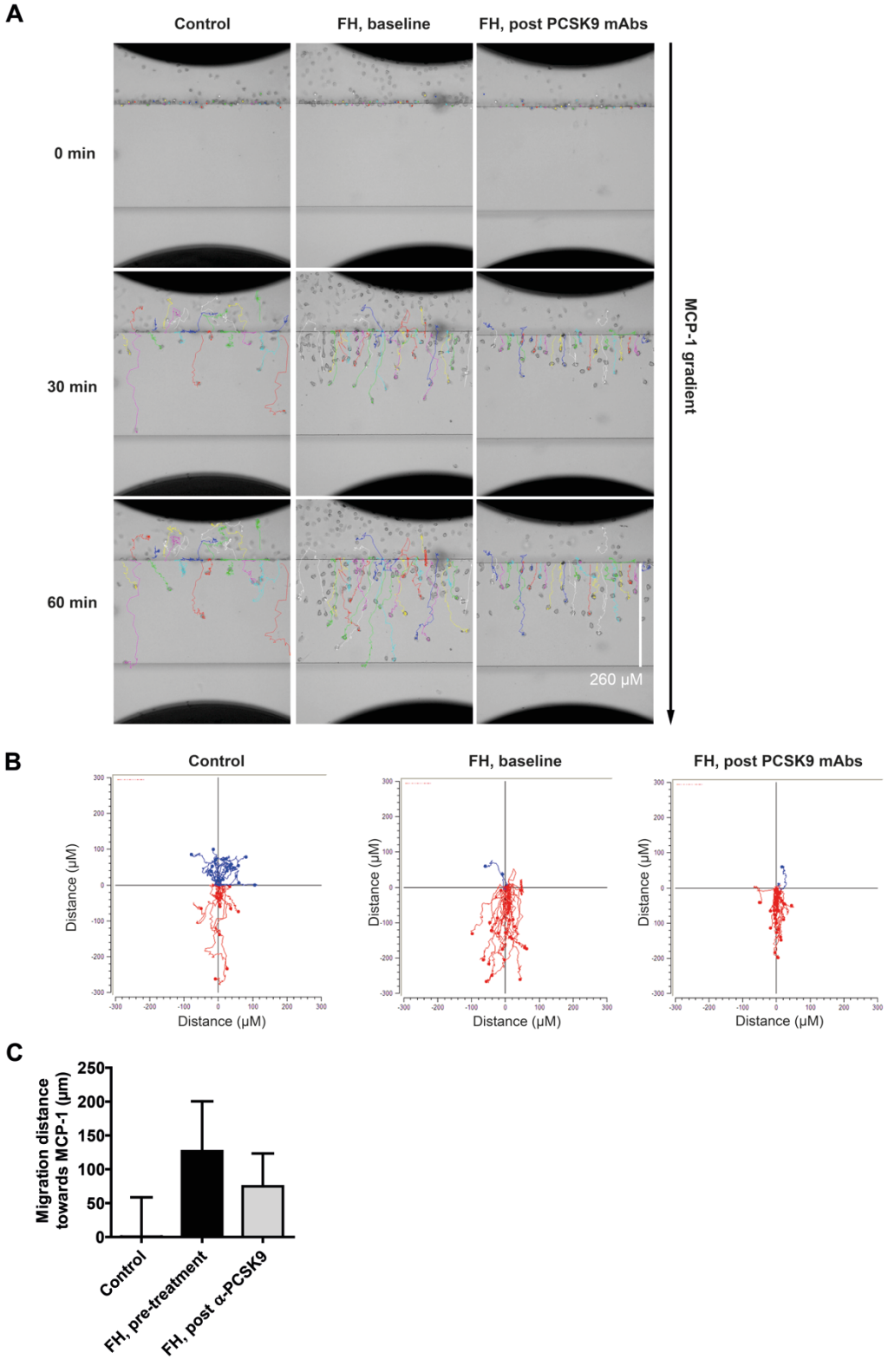
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5