NIBR/TM
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Leqvio A Novartis Cholesterol-Lowering Drug

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Malaga, Spain

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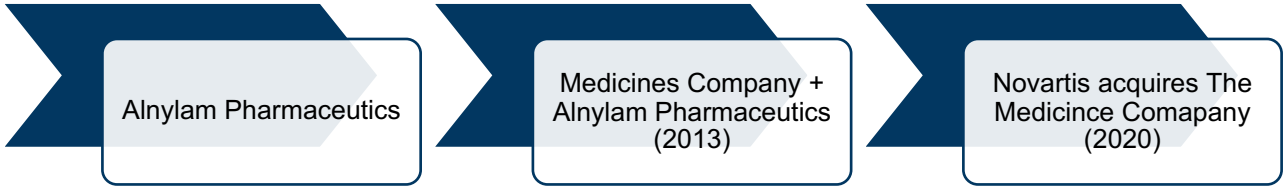
Development Milestones for Leqvio

- Company



- Bioanalysis

- Development activities

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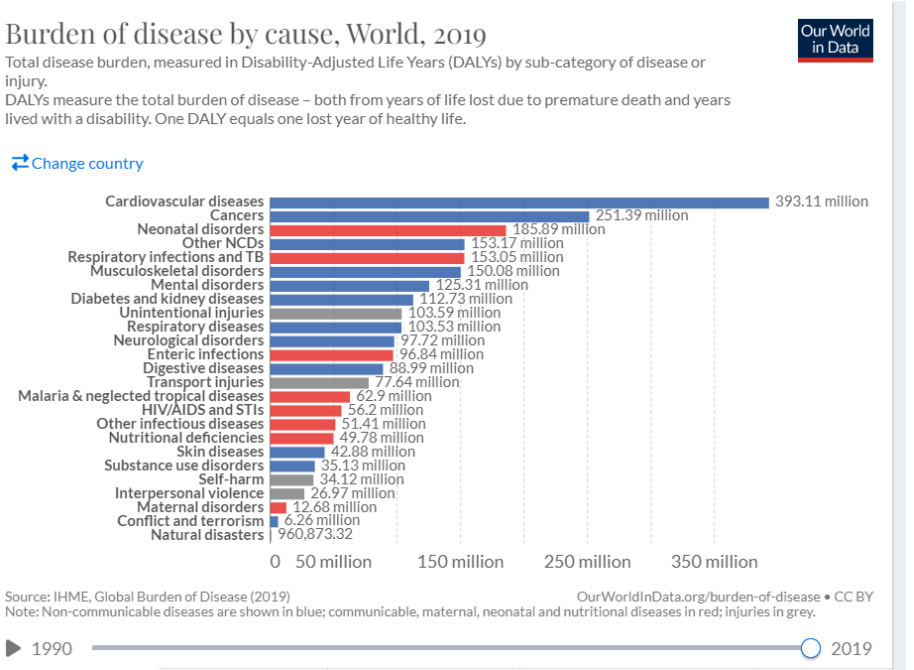
Leqvio/Inclisiran and Atherosclerotic Cardio-vascular Disease

- Clinical data show a strong correlation between Low-density lipoprotein cholesterol (LDL-C) reduction and decline in Atherosclerotic cardiovascular disease (ASCVD)-associated mortality.

- Therefore, it is essential to control circulating LDL-C levels to prevent ASCVD.

- EMA: "*Inclisiran, the active substance in Leqvio, interferes with RNA (genetic material) to limit the production of PCSK9, a protein that can increase levels of LDL-cholesterol ('bad' cholesterol). By preventing PCSK9 production, Leqvio helps to lower LDL-cholesterol levels.*"



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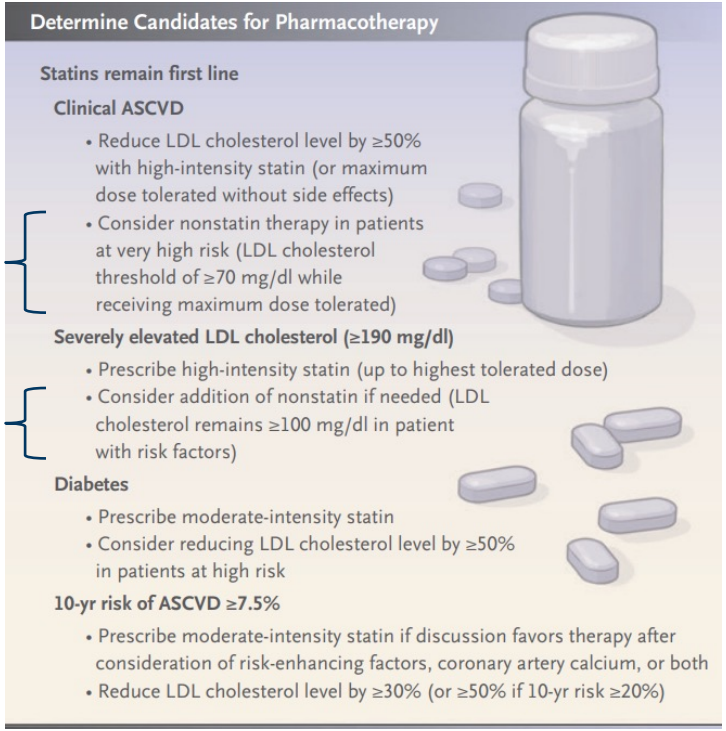
Guidelines for Blood Cholesterol

- Statin therapy is first-line treatment for primary prevention of ASCVD in patients with high to severely elevated low-density lipoprotein cholesterol levels (≥190 mg/dL) and should be taken daily.
- In EU, Leqvio/Inclisiran has been approved for treating adults with primary hypercholesterolaemia (heterozygous familial and non-familial) or mixed dyslipidaemia.
- The Leqvio/Inclisiran patient population are either statin intolerant or are on the maximum dose of statins but do not benefit sufficiently of lower circulating LDL-C levels.



Erin D. Michos *et al* New Eng.J.Med 381;16 Oct 17 2019

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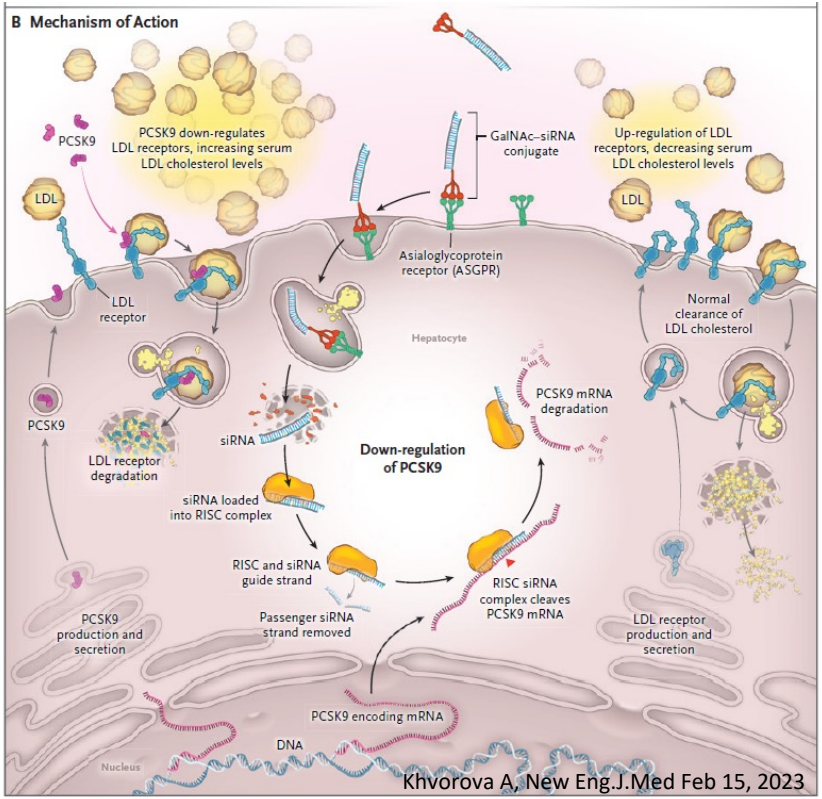
Mechanism of Action

- Leqvio/Inclisiran targets the proprotein convertase subtilisin–kexin type 9 (PCSK9) enzyme.
- By PCSK9 mRNA degradation, PCSK9 translation is prevented
- As a consequence, the expression of the LDL-C receptor is enhanced resulting in lower circulating LDL-C levels
- Inclisiran has the benefit of far lower dose frequency (twice yearly) compared to treatments with monoclonal antibodies to PCSK9 (administered every 2–4 weeks) or statins with half-lifes of 1-3h or 14-19h.



Khvorova A, New Eng.J.Med Feb 15, 2023

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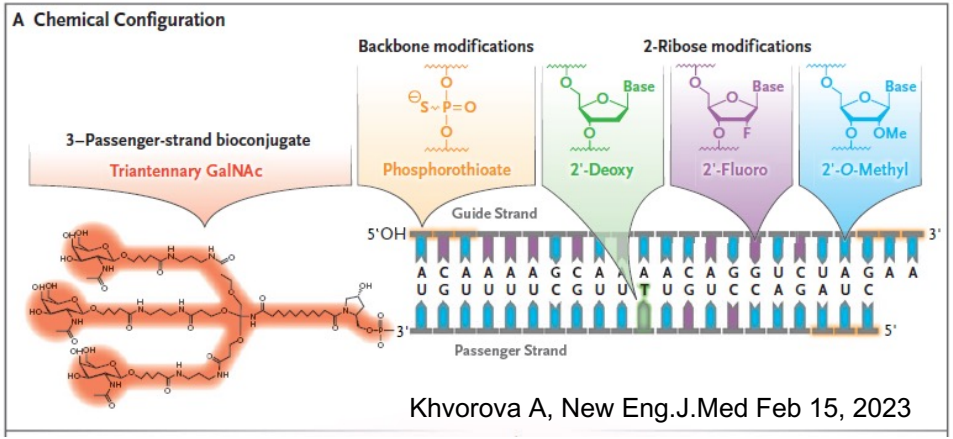
Chemical Structure

- Inclisiran consists of a guide and passenger strand, the nucleotides are modified to improve compound stability (one 2′-deoxy, eleven 2′-fluoro, and thirty-two 2′-*O*-methyl modified nucleotides)
- Termini of the duplex are modified with phosphorothioates, and the 3′ end of the passenger strand is functionalized with triantennary GalNAc.
- The triantennary GalNAc conjugate is specifically recognized by the asialoglycoprotein receptor (ASGPR) that is highly expressed on the surface of liver hepatocytes, the cell type primarily responsible for cholesterol clearance.



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The combination of the 2′-fluoro and 2′-*O*-methyl modifications allows for substantial compound stabilization.

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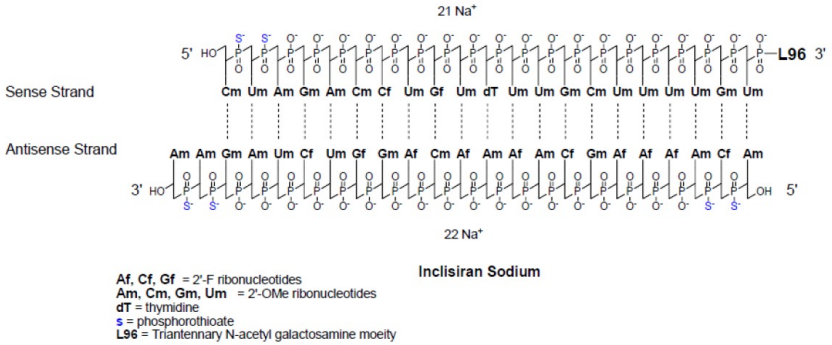
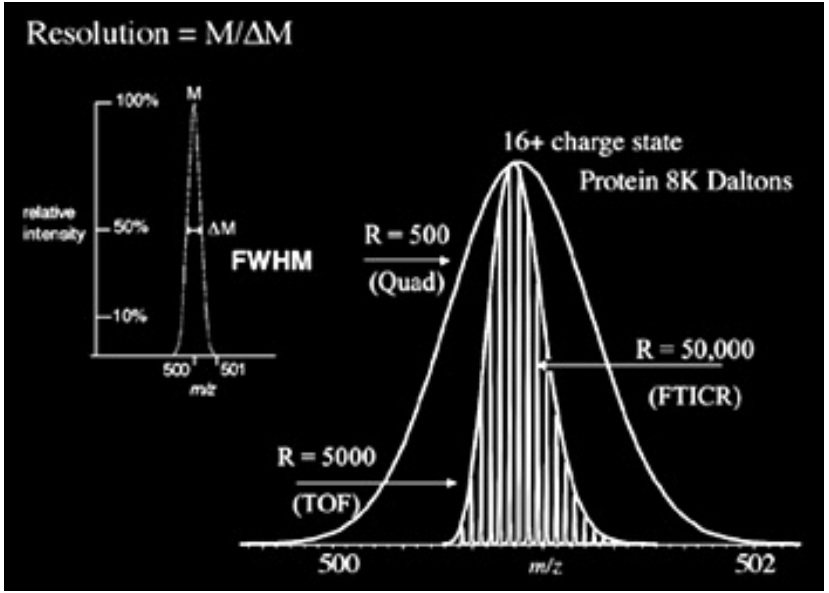
Mass Resolution

- QPS developed a HPLC HRMS method on a Triple TOF 5600 MS



Assessment report, Procedure No. EMEA/H/C/005333/0000 (EMA)

- When using triple-quadrupole mass spectrometry for quantification of siRNAs, data interpretation is difficult due various chemical modifications of synthetic siRNAs to prolong the half-life of the drug in circulation
- High-resolution accurate mass measurement becomes an attractive approach for distinguishing the ions that differ by less than 1 Da in molecular weight



Mass Spectrometry: Basics (scripps.edu)

Compound Name	Duplex Number	Molecular Weight	Strand ID	Single Strand Number	Identity by IPRP-HPLC-ESI-MS
ALN-PCSSC	ALN-60212	16337 Da	Sense	A-122088	8640 Da
			Antisense	A-120190	7697 Da

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HPLC TOF Mass Spectrometry Method

- Plasma samples were processed by solid phase extraction, analyzed using reversed-phase HPLC Triple TOF 5600 MS detection.
- Accurate mass of ten ions for each strand of Inclisiran, antisense (A-120190) and sense (A-122088), and each strand of IS ALN-60519, antisense (A-122227) and sense (A-122230), were monitored in the negative ion mode.
- The peak area for the analyte or IS was the sum of the response from the respective ten ions.
- The peak area ratios of the two single strands were used to construct two separate standard curves using 1/x2 weighted quadratic regression analysis, resulting in two distinct sets of validation data.
- For 100 uL of human plasma a LLOQ of 10 ng/mL was validated using small molecules guidelines. The linear range was 10-10 000 ng/mL.
- PK assay transfer to Novartis qualified vendor (LabCorp). QPS MVR was shared.

How to report the data? A selection criteria was defined by QPS.

<u>Reporting result based on ratio:</u>

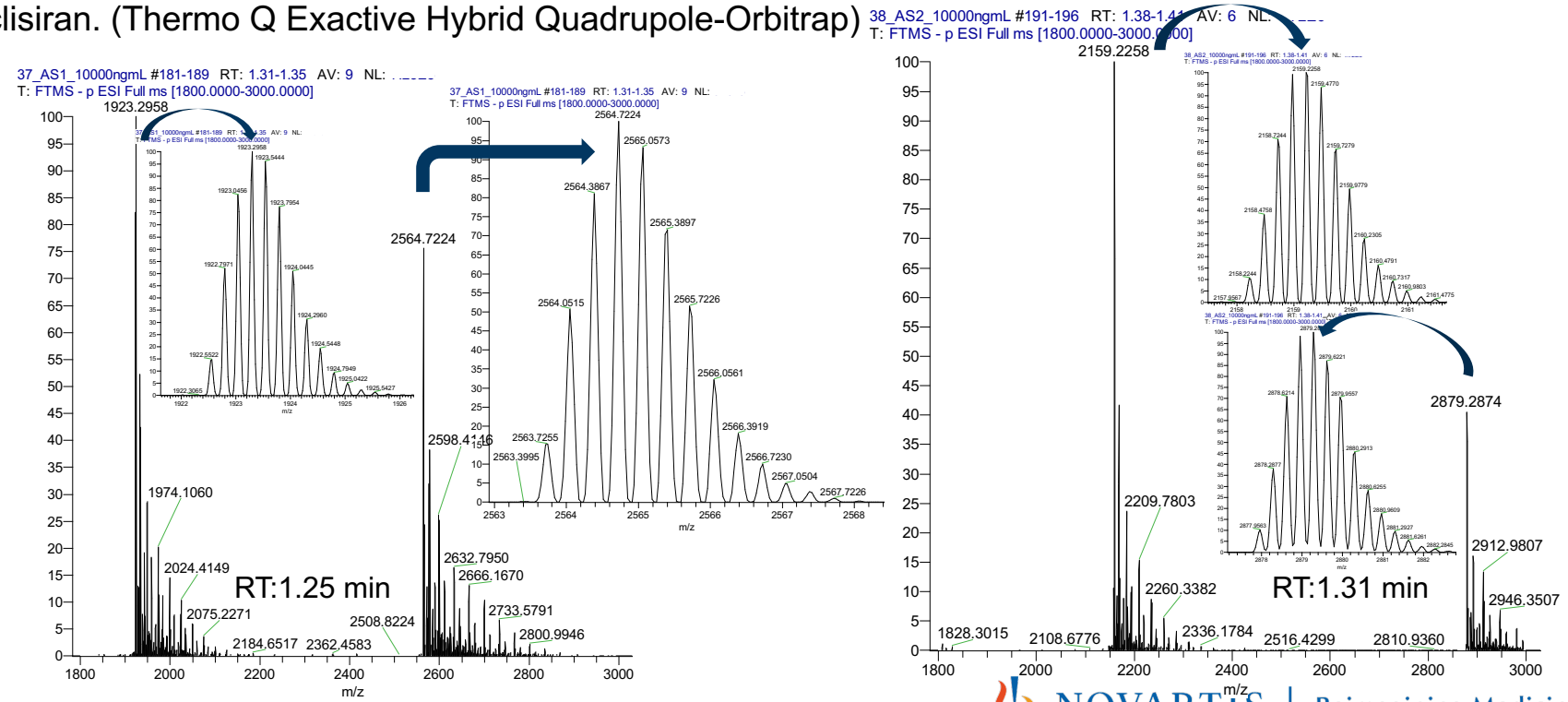
1) [Anti-sense]/Sense] ≥ 1, [Sence] will be reported

IF

2) [Anti-sense]/Sense] ≤ 1, [Anti-sense] will be reported

High Resolution Mass Spectrometry

- The 3rd and 4th changes states of the anti-sense and sense strands were selected for the quantification of Inclisiran. (Thermo Q Exactive Hybrid Quadrupole-Orbitrap)



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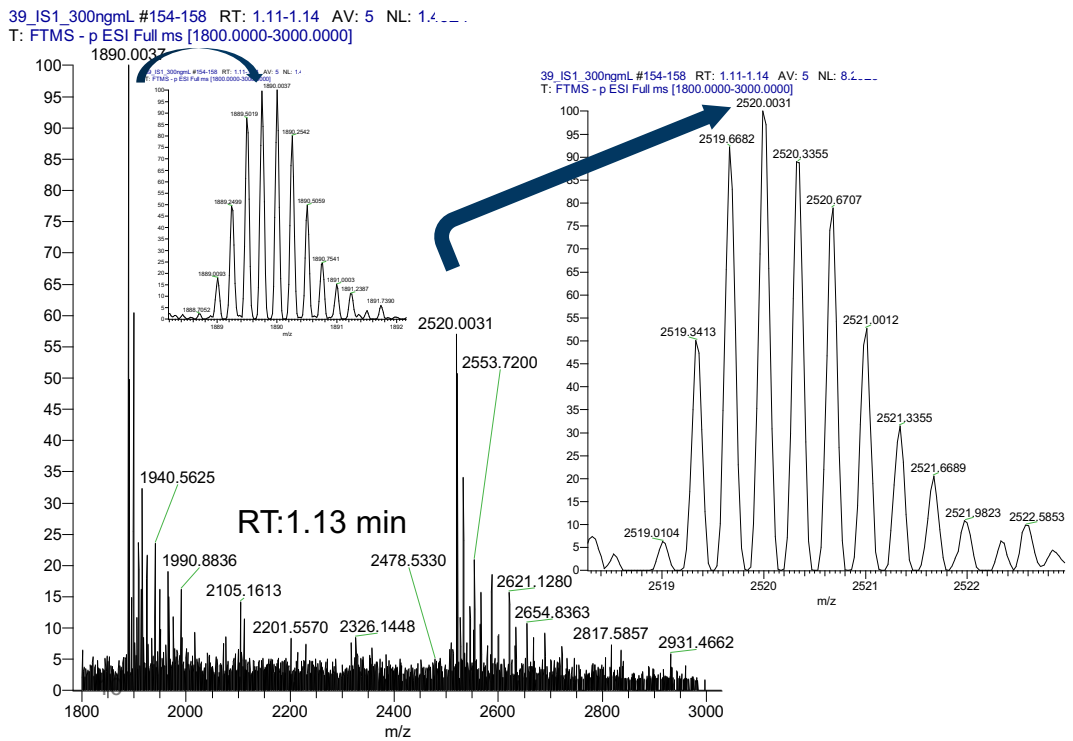
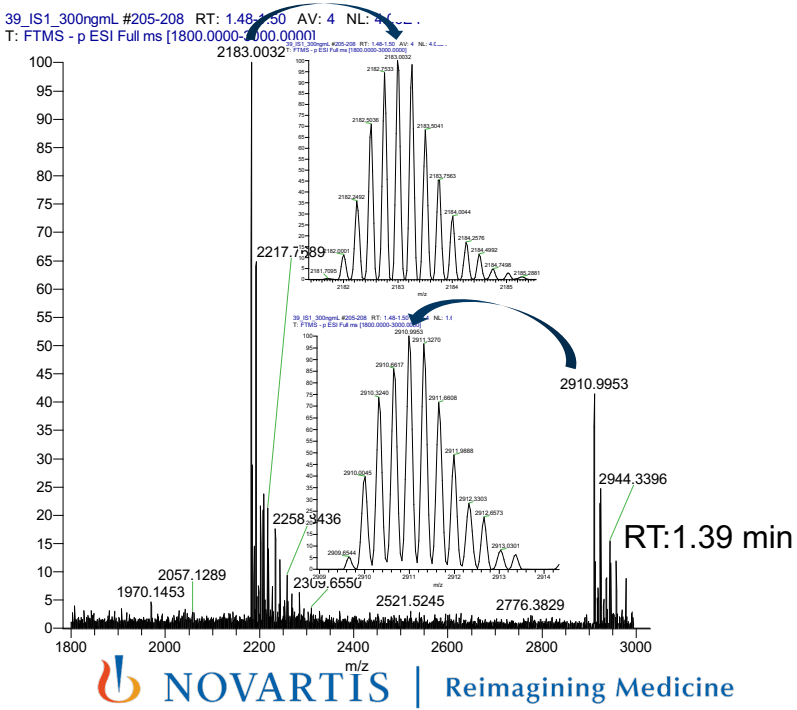
High Resolution Mass Spectrometry

- The 3rd and 4th changes states of the corresponding Internal Standards (analogues) were selected for the quantification of Inclisiran. Thermo Q Exactive Hybrid Quadrupole-Orbitrap





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Inclisiran PK Assay Transfer

- Ethnic sensitivity studies were conduced (China and Japan), and the same dosing regiment was used as in previous studies.
- When analysing the study samples and comparing the results a 2-fold decrease in Cmax and exposure compared to previous studies.
- How come?
- Set up a confidentiality agreement with QPS to discuss.
- We realized that QPS had used Inclisiran as reference and not the anti-sense and sense strands as standards.

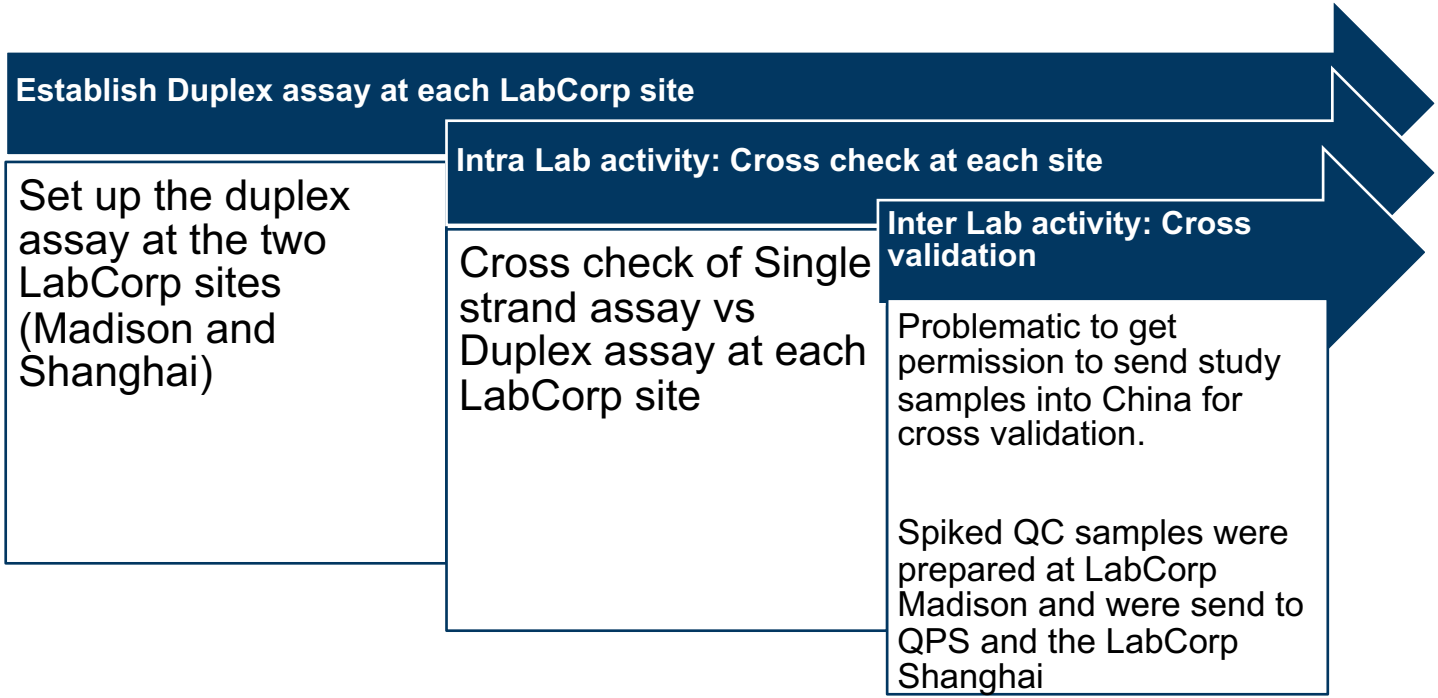
Table 5 **Back-Calculated Concentrations (ng/mL) of ALN-PCSSC (A-122088) Calibration Standards in K_2EDTA Human Plasma**

Run Date	Run ID	10.000	20.000	100.000	300.000	1000.000	3000.000	9000.000	10000.000

Compound Name	Duplex Number	Molecular Weight	Strand ID	Single Strand Number	Identity by IPRP-HPLC-ESI-MS
ALN-PCSSC	ALN-60212	16337 Da	Sense	A-122088	8640 Da
			Antisense	A-120190	7697 Da

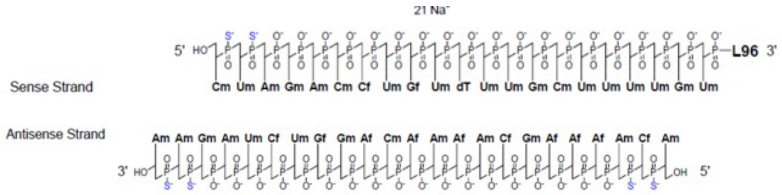
- Based on the molar ratio and the molecular weight, the single strand concentration were recalculated into the corresponding duplex concentration (see back-up slide). Measured concentrations matched with legacy data

Bioanalytical Activities at LabCorp

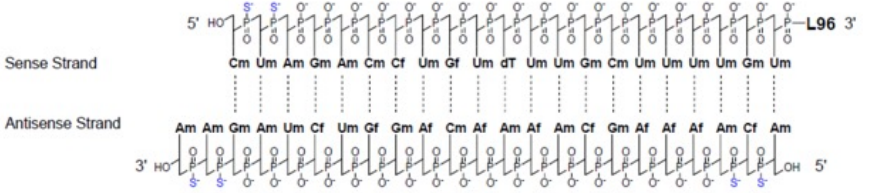


Cross check of two PK assays

Standards and QCs: Sense and Anti-sense



Standard and QCs: Inclisiran



- Prepared Inclisiran QCs (n=6, duplex), measured the QCs samples with the two PK assays in each lab

LabCorp (Madison)

Comparison between two assays

sample name	Mean value in duplex assay	Mean value in single strand assay	Relative difference%
HQC	8365	10040	18.2%
MQC	5607	6162	9.4%
LMQC	405	405	0.1%
LQC	31	30	-2.8%

LabCorp (Shanghai)

Comparison between two assays

sample name	Mean value in duplex assay	Mean value in single strand assay	Relative difference%
HQC	3400	3710	8.7
MQC	1660	1710	3.0
LMQC	490	505	3.0
LQC	26.8	29.2	8.6

- Average Cmax was approximately at 500 ng/mL
- Conclusion: The assay are comparable in the concentration range measured in the study (300mg 2*year)

Cross validation of three PK assays

For Novartis studies, the reference laboratory was LabCorp Madison. All PK samples collected outside of China were measured at Madison. PK sample collected in China were measured at LabCorp Shanghai. All legacy studies were measured at QPS.

Compared cross validation data between QPS and Labcorp-Madison lab

QC level	Final reported KJX839 duplex Concentration (ng/mL) from QPS	Final reported KJX839 duplex Concentration (ng/mL) from Madison	Relative Difference (%)
HQC	7550	8280	9.2
MQC	4600	5610	19.8
LMQC	392	383	-2.3
LQC	29.0	26.5	-9.0

Compared cross validation data between Labcorp-Shanghai and Labcorp-Madison lab

QC level	Final reported KJX839 duplex Concentration (ng/mL) from Shanghai	Final reported KJX839 duplex Concentration (ng/mL) from Madison	Relative Difference (%)
HQC	8080	8280	2.4
MQC	5430	5610	3.3
LMQC	389	383	-1.6
LQC	29.1	26.5	-9.4

Conclusions

- Leqvio/Inclisiran is a double stranded small-interfering RNA (siRNA) consisting of a sense and anti-sense strand, both are modified to improve stability.
- Ultrahigh Pressure Liquid Chromatography/High-Resolution Accurate Mass-MS (UPLC/HRMS) methods were transferred and validated according to current guidelines for small molecules.
- UPLC/HRMS result in improved selectivity, better signal-to-noise ratio and greater sensitivity. For 100 uL of human plasma a LLOQ of 10 ng/mL was validated.
- Successful cross validations ensured that the clinical development program with a global footprint was supported by several bioanalytical sites.

Thank you

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Recalculation of the concentration

- Concentration (ng/mL), molecular weight (g/mol), volume = 1 mL
- Back calculate antisense to duplex concentration
- C1: 10.2 ng/mL of antisense mw 7697 (g/mol)→ 10.2 *10 $^{-10}$ / 7679 mol antisense
- 1 mol antisense = 1mole duplex
- Therefore, we have: (10.2 *10 $^{-10}$ / 7679) * 16337 g duplex = 21.2 ng/mL duplex

Compound Name	Duplex Number	Molecular Weight	Strand ID	Single Strand Number	Identity by IPRP-HPLC-ESI-MS
ALN-PCSSC	ALN-60212	16337 Da	Sense	A-122088	8640 Da
			Antisense	A-120190	7697 Da