Insights into the dynamics of PCSK9/LDLR interaction: effect of gain-of-function mutations and pH-dependent affinity

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OVERVIEW

An ELISA (Enzyme-linked immunosorbent assay) was designed to evaluate the effect of candidate drugs on PCSK9/LDLR binding. We optimized experimental parameters and buffer compositions to detect conformational effects of pH and PCSK9 mutations. We observed that:

- PCSK9 binding affinity for LDLR was higher at endosomal pH 6.0 than at neutral pH 7.4
- Gain-of-function mutations of PCSK9 increased binding affinity for LDLR at both endosomal and neutral pH, and the order of potency at acidic and neutral pH was WT < D374T << D374Y
- AlphaLISA® assays and Bio-layer interferometry (BLI) showed similar trends
- Neutralizing antibodies against PCSK9 blocked PCSK9/LDLR binding

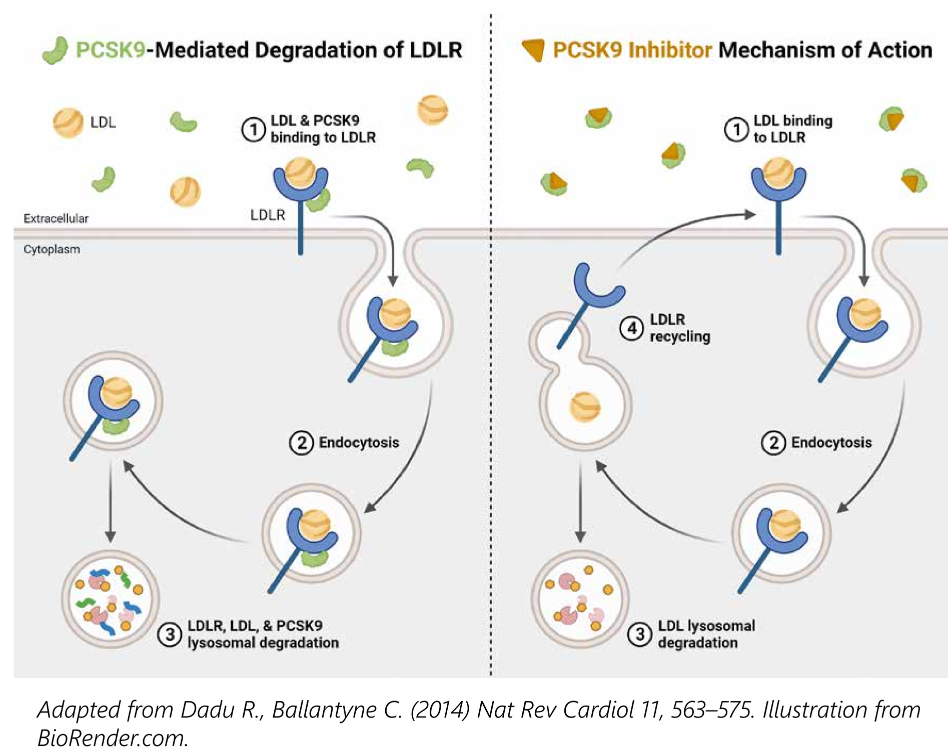
In summary, PCSK9/LDLR assays can assess pH-dependent differences in LDLR binding affinities of PCSK9 wild-type and mutant proteins and allow for a comprehensive understanding of binding dynamics.

BACKGROUND

Cholesterol is vital for the maintenance of cell membrane structures, the synthesis of steroid hormones, and for cellular metabolism. Its distribution in the body occurs through lipoprotein complexes, notably low and high density lipoproteins (LDL and HDL). LDL enters cells by binding to a low-density lipoprotein receptor (LDLR) present on the cell surface. Once internalized, the LDL/LDLR complex undergoes intracellular sorting within endosomes, where the acidic environment facilitates dissociation. LDL is processed in the lysosomes, releasing cholesterol and other lipids for use in the cytosol. Meanwhile, LDLR is recycled to the cell surface, primed for another cycle of LDL binding. Hence, LDLR functions to clear LDL from the circulation.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a liver-secreted protein that regulates circulating cholesterol levels by decreasing cell surface expression of LDLR. PCSK9 acts by binding to LDLR and inducing conformational changes that earmark it for degradation rather than recycling. This reduces LDLR availability, leading to high LDL levels in the bloodstream.

Gain-of-function (GOF) mutations in PCSK9, such as D374T or D374Y, elevate LDL levels and increase risk of cardiovascular diseases, while loss of function mutations confer protection. Anti-PCSK9 antibodies such as Alirocumab and Evolocumab counteract the detrimental impact of GOF mutant PCSK9 by preventing PCSK9 binding to LDLR, increasing the number of functional LDLRs, and lowering LDL levels in the blood.



Adapted from Dadu R., Ballantyne C. (2014) Nat Rev Cardiol 11, 563–575. Illustration from BioRender.com.

pH-DEPENDENT BINDING OF PCSK9 / LDLR

Chemiluminescent ELISA Principle and Methods

Experiments were performed in duplicates in white 96-well plates. The recombinant LDLR ectodomain was diluted in Phosphate Buffered Saline (PBS) and coated on the plate at 100 ng/well overnight at 4°C. After blocking for 1 hour at room temperature (RT), Biotin-PCSK9 proteins were added together with serial dilutions of anti-PCSK9 neutralizing antibody (BPS Bioscience #71207) or PCSK9 inhibitor Pep2-8 (MedChemExpress #HY-P276) in assay buffer at pH 6.0 or pH 7.4, and incubated for 2 hours at RT prior to washing. Streptavidin-HRP was incubated with the protein complexes for 1 hour at RT, washed, and the chemiluminescent HRP substrate was added. Signal was read in a luminescent plate reader, and blank value (condition without Biotin-PCSK9) was subtracted from all other values.

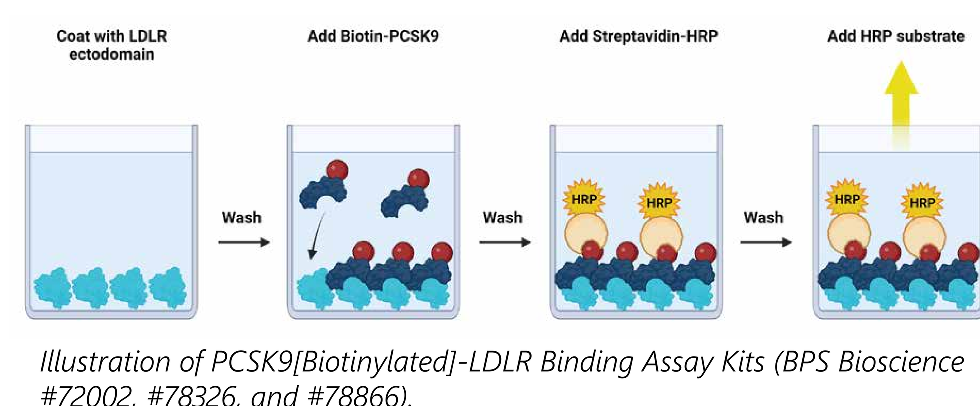


Illustration of PCSK9[Biotinylated]-LDLR Binding Assay Kits (BPS Bioscience #72002, #78326, and #78866).

Results

The effect of GOF mutations D374T and D374Y on PCSK9 binding to the ectodomain of LDLR was compared at endosomal pH (pH 6.0) and physiological pH (pH 7.4). PCSK9 showed higher affinity for LDLR at acidic pH compared to neutral pH, indicating that PCSK9 binds more avidly to the LDLR protein in the lysosomal/endosomal compartments. Notably, the PCSK9 (D374T) and PCSK9 (D374Y) GOF mutants had higher affinity for LDLR than wild-type PCSK9 at both pH (pH 7.4 and pH 6.0). The D374T mutation increased PCSK9-LDLR binding by ~2-3-fold, while D374Y led to a ~40-fold increase in LDLR binding compared with wild type PCSK9.

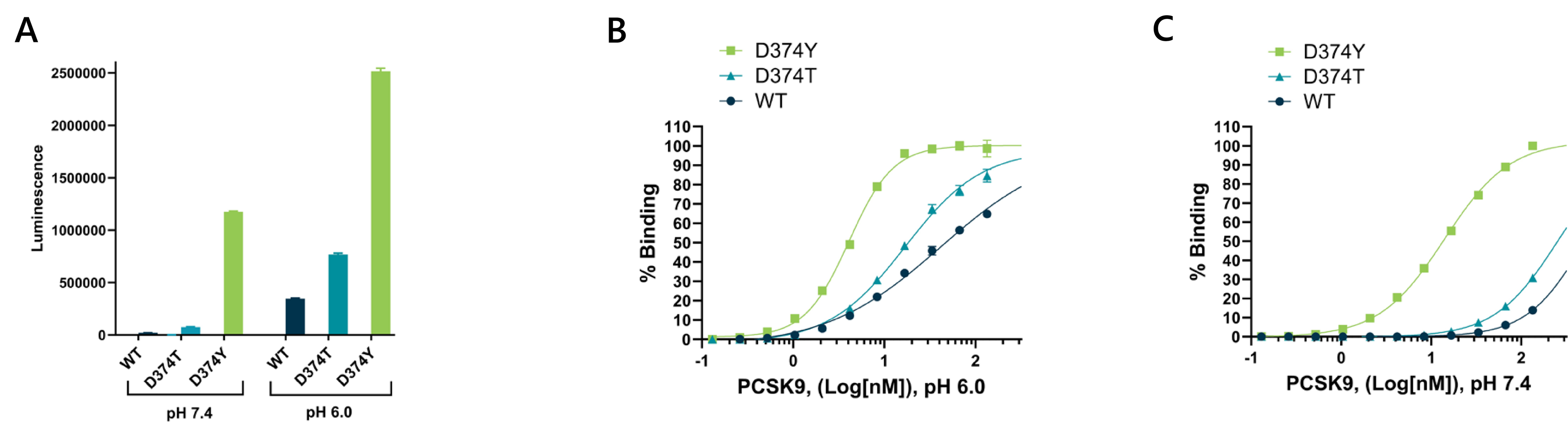


*Figure 1: PCSK9 binding to LDLR ectodomain analysed by ELISA. **(A)** Raw luminescence data from ELISA measuring the binding of LDLR to PCSK9 WT or mutant at pH 6.0 and pH 7.4. **(B, C)** Increasing concentrations of PCSK9 wild-type or mutant were incubated with the pre-coated LDLR ectodomain at pH 6.0 **(B)** and at pH 7.4 **(C)**. Results are expressed as percent of binding (maximum binding set to 100%). The table shows EC_{50} in each condition, determined by curve fitting using Prism software v 11.0.*

Protein	EC_{50} at pH 6.0	EC_{50} at pH 7.4
PCSK9 WT	~41 nM	~506 nM
PCSK9 D374T	~17 nM	~252 nM
PCSK9 D374Y	4 nM	~14 nM

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ORTHOGONAL VALIDATION

AlphaLISA® Methods

Experiments were performed in duplicates in Optiplate-384 (PerkinElmer #6007290). Purified proteins were thawed on ice and diluted in the assay buffer at the indicated pH. Biotinylated or His-tagged PCSK9 enzymes were incubated with FLAG-tag LDLR ectodomain, with or without serial dilutions of a neutralizing compound of interest in assay buffer (pH 6.0 or pH 7.4) for 1 hour at room temperature (RT). AlphaLISA® anti-FLAG Acceptor beads and either Streptavidin (Figure 2A) or Nickel (Figure 2B) Donor beads were added to the proteins for 1 hour at RT with slow shaking prior to measurement. *AlphaLISA® is a registered trademark of PerkinElmer, Inc.*

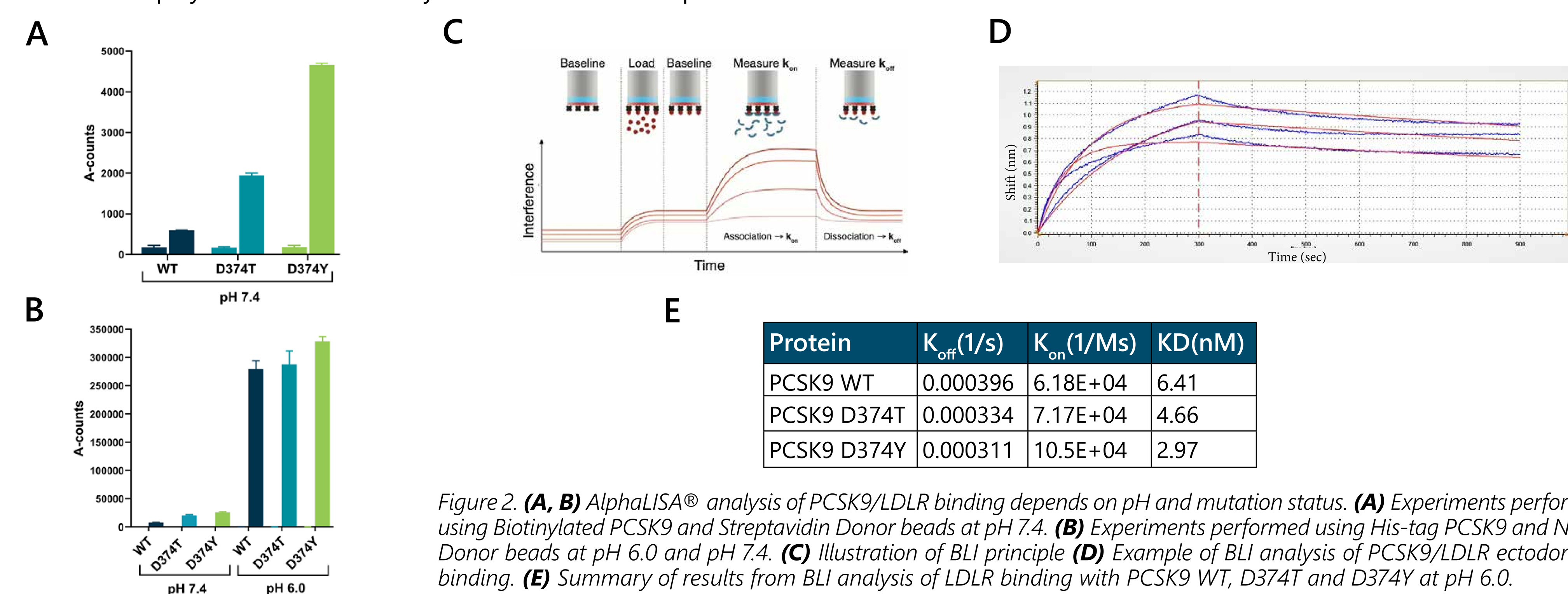
Anti-FLAG Acceptor Bead — $^1O_2$ — FLAG — LDLR — PCSK9 — Biotin — Streptavidin Donor Bead

BLI

Bio-layer interferometry (BLI) experiments were performed using a Gator Prime Device. WT or mutant PCSK9 proteins (biotin-labeled) were loaded at a pre-determined concentration of 50 nM onto streptavidin XT probes (Gator Bio #160029). A kinetic assay was performed with increasing amounts of LDLR in the association step, and a dissociation step in assay buffer (pH 6). K_{on} and K_{off} rates were determined by Gator Bio's curve-fitting software.

Results

AlphaLISA® assays and BLI corroborated the trends observed by ELISA. Indeed, the affinity of PCSK9-WT or GOF for LDLR was enhanced at acidic pH compared to neutral pH. In both pH conditions, PCSK9 mutant D374Y had greater affinity for LDLR than mutant D374T and than WT, whereas PCSK9-WT displayed the lowest affinity for LDLR of the three proteins.



Protein	K_{off}(1/s)	K_{on}(1/Ms)	KD(nM)
PCSK9 WT	0.000396	6.18E+04	6.41
PCSK9 D374T	0.000334	7.17E+04	4.66
PCSK9 D374Y	0.000311	10.5E+04	2.97

*Figure 2. **(A, B)** AlphaLISA® analysis of PCSK9/LDLR binding depends on pH and mutation status. **(A)** Experiments performed using Biotinylated PCSK9 and Streptavidin Donor beads at pH 7.4. **(B)** Experiments performed using His-tag PCSK9 and Nickel Donor beads at pH 6.0 and pH 7.4. **(C)** Illustration of BLI principle **(D)** Example of BLI analysis of PCSK9/LDLR ectodomain binding. **(E)** Summary of results from BLI analysis of LDLR binding with PCSK9 WT, D374T and D374Y at pH 6.0.*

POTENCY OF NEUTRALIZING ANTIBODY OR PEP2-8

A neutralizing anti-PCSK9 antibody and inhibitor Pep2-8 blocked the binding of LDLR to PCSK9 wild-type or mutant, both at pH 6.0 and at pH 7.4. Our results are consistent with published data (Fisher *et al.* 2007, *J. Biol. Chem.* 282: 20502-12) and emphasize the utility of these assays to assess PCSK9/LDLR binding using clinically relevant mutants of PCSK9.

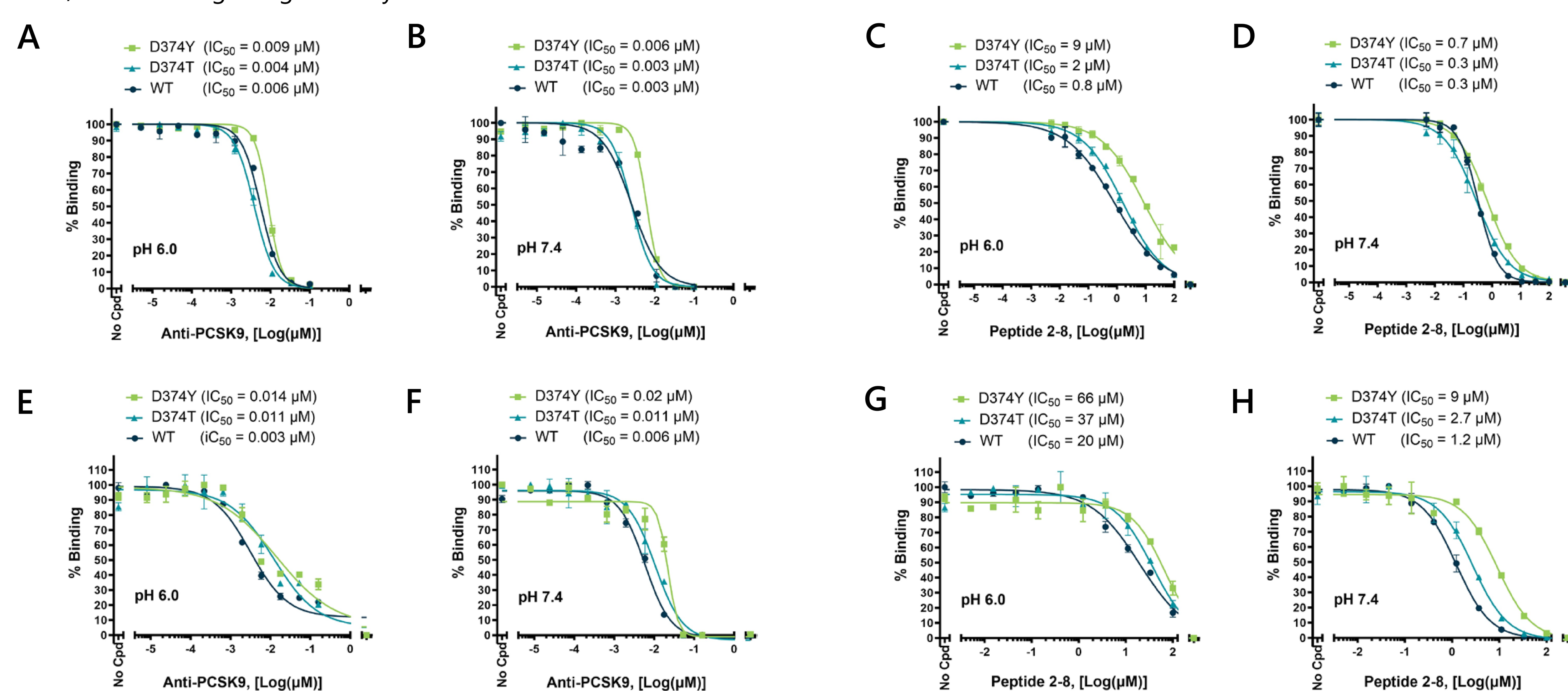


*Figure 3: Blocking of PCSK9/LDLR binding by neutralizing antibodies or Pep2-8 measured using ELISA (top panels A-D) or AlphaLISA® (bottom panels E-H). Binding was measured in the presence of increasing concentrations of a neutralizing antibody (BPS Bioscience #71207) **(A, B, E, F)** or Pep2-8 **(C, D, G, H)** at neutral or acidic pH as indicated. Results are expressed as percent of binding (maximum binding set to 100%).*

CONCLUSION

In summary, our experimental findings are consistent with existing literature on PCSK9 mutations and bear relevance to the clinical implications associated with gain-of-function mutations in PCSK9, demonstrating the utility of these assays. pH-sensitive assays to measure LDLR binding to various forms of PCSK9, both wild-type and mutant, support drug discovery and developmental endeavors by providing physiologically relevant insights. For example, optimizing drug candidates to function effectively across a range of pH conditions enhances their potential clinical applicability. Dysregulation of PCSK9-LDLR interaction is implicated in cardiovascular disease, and understanding how binding affinity is altered at specific pH levels could be relevant to diseases associated with abnormal pH in cellular compartments. Thus, measuring PCSK9-LDLR binding at different pH levels is crucial for understanding the physiological relevance, optimizing drug development, unraveling intracellular trafficking mechanisms, exploring disease mechanisms, and gaining basic biological insights into this important molecular interaction.