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N-terminal mutants of human apolipoprotein A-I: structural perturbations associated to protein misfolding

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5 Short title: Structural perturbations involved in amyloidosis from N-terminal mutants of
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21

22 **Abstract**

23 Since the early description of different human apolipoprotein A-I variants
24 associated to amyloidosis, the reason that determines its deposition inducing organ
25 failure has been under research. To shed light into the events associated to protein
26 aggregation, we studied the effect of the structural perturbations induced by the
27 replacement of a Leucine in position 60 by an Arginine as it occurs in the natural
28 amyloidogenic variant (L60R). Circular dichroism, intrinsic fluorescence measurements
29 and assays of binding to ligands indicate that L60R is more unstable, more sensitive to
30 proteolysis and interacts with sodium dodecyl sulfate (a model of negative lipids) more
31 than the protein with the native sequence and other natural variant tested, involving a
32 replacement of a Tryptophan by an Arginine in the amino acid 50 (W50R). In addition,
33 the small structural rearrangement observed under physiological pH leads to the release
34 of tumor necrosis factor α and interleukin-1 β from a model of macrophages. Our results
35 strongly suggest that the chronic disease may be a consequence of the loss in the native
36 conformation which alters the equilibrium among native and cytotoxic proteins
37 conformation.

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41 **Introduction**

42 Human apolipoprotein A-I (apoA-I) is the main protein associated to high
43 density lipoproteins (HDL). It is synthesized in liver and intestine and both the liver and
44 the kidney are the major sites of its catabolism. Its multiple functions as lipid transport,
45 endothelial homeostasis and inhibition of inflammatory pathways [1][2][3] could
46 however be counter balanced by the presence of single point mutations which could, by
47 not yet completely known pathways, induce its misfunction, increased clearance rate or
48 its tendency to aggregate [4][5]. Hereditary apoA-I amyloidosis has been described
49 since 1969, and is characterized by specific deposits of natural variant proteins within
50 organs, in a pattern which is dependent on the mutation in the protein sequence. From
51 the more than 20 known natural variants, almost half result from substitutions between
52 amino acids 26 and 107, and involve with different severity hepatic and kidney failure.
53 Instead, a “hot spot” affecting residues 173-178 was described, in which variants are
54 especially associated to cardiac, skin and testis damage. The reasons for the different
55 deposit patterns are still unknown but may be the consequence of long term seeding of
56 misfolded proteins that yield a final fibrillar conformation.

57 The first identified apoA-I natural mutant was the result of a substitution of a
58 Glycine by an Arginine in position 26 from the native sequence (Gly26Arg, in the
59 abbreviated nomenclature G26R), inducing in patients peripheral neuropathy, peptic
60 ulcer, and nephropathy [6]. The next mutations described in the N terminus of apoA-I
61 were Trp50Arg (W50R) [7] and Leu60Arg (L60R) [8]. These variants show similarities
62 with G26R: in each, a neutral residue is replaced by an Arginine thus increasing by one
63 the positive net charge, and amyloid fibrils isolated from the tissues consist of the N-
64 terminal fragments of the variant apoA-I.

65 As a difference from other hereditary amyloidosis, in which renal failure occurs
66 mainly due to glomerular protein deposits [9][10], apoA-I associated disease is mostly
67 characterized by amyloid retention in the medullary interstitium and/or vasculature,
68 which is probably a reason for its misdiagnosis [11][12]. Only rare exceptions were
69 observed within a few mutants including W50R, in which amyloid deposits were found
70 in glomeruli, either confined [13], or expanded to medulla. Previous works from our
71 and other groups have demonstrated that the single point mutations described for apoA-
72 I decrease the marginal protein stability and elicit the tendency to aggregate; even
73 though the conformational shift in the variants is usually subtle under physiological pH
74 and low concentration, it could also induce alterations of the binding to ligands or the
75 eliciting of pro-inflammatory cellular events [14][15]. In order to extend the knowledge
76 about structural motifs that could be involved in apoA-I aggregation and pathogenicity,
77 we herein studied and described the structural initial events that could result in high
78 yield of misfolded conformations in the pro amyloidogenic variant L60R. We compared
79 this mutant behavior with the Wt and with W50R, which is involved in renal
80 amyloidosis but with a relative different aggregation clinical profile. This last mutant
81 served as good comparison as some structural characterizations were already performed
82 by Gursky and colleagues[16][17].

83

84

85 **Materials and methods**

86 **Materials**

87 Reagents purchased from Sigma-Aldrich (St Louis, MO) comprise the
88 following: matrix metalloproteinase-12 (MMP-12, Catalytic Domain), Phorbol 12-
89 myristate 13-acetate (TPA), Trypsin, Guanidine hydrochloride (GdmCl), sodium

90 dodecyl sulfate (SDS), thioflavin T (ThT), polymixin B; 1,2-dimyristoylsn-glycero-3-
91 phosphatidylcholine, (DMPC) was from Avanti Polar Lipids (Alabaster, AL). IMAC
92 Sepharose 6 Fast Flow Resin was acquired from GE Healthcare Bio-Sciences AB,
93 Uppsala, Sweden). The glycosamine glycan Heparin from bovine intestinal mucosa
94 (average molecular weight 15 kDa) was from Northia (BA, Argentina). From Invitrogen
95 (Carlsbad, CA) we obtained 4,4'-dianilino-1,1'-binaphthyl- 5,5'-disulfonic acid,
96 dipotassium salt (Bis-ANS). Isopropyl- β -D-thiogalactoside (IPTG) was purchased to
97 Thermo Scientific (Waltham, MA). All other reagents were acquired with the highest
98 analytical grade.

99

100 **Methods**

101 **Cloning, expression, and purification of wild-type (Wt) W50R and** 102 **L60R mutants of apoA-I**

103 A cDNA template containing human Wt apoA-I sequence was used in order to
104 introduce the required single point mutations. This construct was inserted into a pET-30
105 plasmid (Novagen, Madison, WI), allowing the expression and purification of the apoA-
106 I variants fused to an N-terminal His-Tag peptide [18]. The mutants W50R and L60R
107 were obtained by the Quick change method (Stratagene, La Jolla, CA). Primers were as
108 follows:

109 For W50R: Sense 5'-agctccttgacaacagggacagcgtgacc-3'

110 Antisense 5'-ggtcacgctgtccctgttgaaggagct-3'

111 For L60R Sense 5'-caccttcagcaagcggcgcaacagctcg-3'

112 Antisense 5'-cgagctgttcgcccgttgctgaaggtg-3'

113 In order to further remove this N terminal peptide, an Asp—Pro sequence
114 involving amino acid residues 2 and 3 was previously generated to allow specific
115 chemical cleavage [19][20]. Proteins were expressed in BL 21E *Coli* strands following
116 induction with IPTG and purified by elution through Nickel affinity columns (GE
117 Healthcare Bio-Sciences AB, Uppsala, Sweden). The His Tag was efficiently removed
118 by cleavage with 45% formic acid at 45°C for 5 h, following exhaustive dialysis against
119 buffer Tris 20 mM, NaCl 150 mM, pH 8.0). A second metal affinity chromatography
120 step was run to separate the final pure protein fraction.

121

122 **Mass Spectrometry Analysis**

123 Protein digestion and Mass Spectrometry analysis were performed at the
124 Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/CONICET
125 (National Research Council) as follows: Protein samples were reduced with
126 dithiothreitol (DTT) and alkylated with iodoacetamide in Ammonium Bicarbonate 50
127 mM pH 8.0. This protein solution was precipitated with trichloroacetic acid (TCA).
128 Proteins were resuspended in the same buffer, digested with trypsin (Promega V5111),
129 peptides purified and desalted with ZipTip C18 columns (Millipore). The digests were
130 analyzed by nanoLC-MS/MS in a Thermo Scientific Q-Exactive Mass Spectrometer
131 coupled to a nanoHPLC EASY-nLC 1000 (Thermo Scientific). For the LC-MS/MS
132 analysis, approximately 1 µg of peptides was loaded onto a reverse phase column (C18,
133 2 µm, 100 Å, 50 µm x 150 mm) Easy-Spray Column PepMap RSLC (P/N ES801)
134 suitable for separating protein complexes with a high degree of resolution. The MS
135 equipment has a high collision dissociation cell (HCD) for fragmentation and an
136 Orbitrap analyzer (Thermo Scientific, Q-Exactive). XCalibur 3.0.63 (Thermo Scientific)
137 software was used for data acquisition and equipment configuration that allows peptide
138 identification at the same time of their chromatographic separation. Full-scan mass

139 spectra were acquired in the Orbitrap analyzer. Q-Exactive raw data were processed
140 using Proteome Discoverer software (version 2.1.1.21 Thermo Scientific) and searched
141 against apoA-I sequence database downloaded from NCBI (National Center for
142 Biotechnology Information) (www.ncbi.nlm.nih.gov) digested with trypsin with a
143 maximum of one missed cleavage per peptide.

144 The exponentially modified protein abundance index (emPAI) was calculated
145 automatically by Proteome Discoverer software and used to estimate the relative
146 abundance of identified proteins within the sample.

147

148 **Protein structure and chemical stability**

149 ApoA-I Wt, W50R and L60R were diluted in Tris 20 mM pH 7.4 (Tris buffer) or
150 citrate phosphate McIlvaine's buffer(Citrate buffer) pH 5.0 [21]. Variants were taken at
151 0.2 mg/mL (at 25°C). Tryptophan (Trp) intrinsic fluorescence emission spectra was
152 acquired in an SLM4800 spectrofluorometer (ISS Inc, Champaign, IL) upgraded by
153 Olis, setting the excitation wavelength at 295 nm and scanning emission from 310 to
154 400 nm. Solvent exposure of Trp residues was determined as a parameter of protein
155 structural arrangement. ApoA-I variants were diluted to 0.1 mg/mL (in Tris buffer).
156 Quenching of the Trp residues was determined by measuring intrinsic fluorescence
157 following stepwise addition of acrylamide from a concentrated stock solution. The
158 quenching constant K was calculated from a linear plot of the Stern-Volmer equation as:

159

$$160 \quad F_0/\Delta F = 1/(f_a \times K [Q]) + 1/f_a \quad (1)$$

161

162 where f_a is the fraction of the initial fluorescence which is accessible to the quencher, K
163 is the Stern-Volmer quenching constant of the accessible fraction and [Q] is the

164 concentration of the quencher. F_0 is the initial fluorescence in the absence of quencher
165 and ΔF is the remaining fluorescence after the addition of acrylamide at each
166 concentration [22][15].

167 In order to sense hydrophobic pockets within the proteins spatial arrangements,
168 the binding of Bis-ANS was measured following titration of the probe to apoA-I
169 variants. Proteins were set at 0.1 mg/mL in Tris buffer and Bis-ANS added in small
170 amounts from a methanol stock. Both, total intensity and spectral shift were registered
171 by excitation at 395 nm and emission scanned from 450 to 550 nm [15]. Circular
172 dichroism (CD) measurements were acquired on a Jasco J-810 spectropolarimeter. To
173 register far UV spectra (200–290 nm), proteins were diluted in phosphate 10 mM
174 buffer, pH 7.4 to 0.2 mg/mL in a 1 mm path-length cuvette, and three scans at a speed
175 of 20 nm min⁻¹ were registered and averaged to minimize noise signal. Mean residue
176 weight values of 115.5, 115.4 and 115.7 for Wt, were estimated to calculate molar
177 ellipticity as previously described [23]. Near CD spectra were registered under the same
178 conditions but with a final protein concentration of 1.0, 1.5 and 1.5 mg/mL for Wt,
179 W50R, and L60R, respectively.

180 To characterize apoA-I variants' stability respect to the Wt, Trp fluorescence
181 spectra were measured following stepwise additions of increasing amounts of GdmCl
182 [15][14]. As described in our previous works, the free energy of unfolding in the
183 absence of denaturant ($\Delta G^{\circ}H_2O$) was calculated from the shift of the Trp spectral
184 center of mass as a function of GdmCl final concentration assuming the simplest two
185 state process model [18][19].

186 In order to compare the self-association of the proteins in solution, Wt, W50R,
187 and L60R were taken to 0.5 mg/mL in Phosphate Buffer (pH 7.4) and eluted through a
188 Sepharose 200 HR column (Amersham Pharmacia recommended MW 10,000-600,000),

189 at a Flow rate of 0.35 mL/min, connected to a Merck Hitachi Fast Performance Liquid
190 Chromatography equipment. Elution profile was followed by UV detector (280 nm).

191

192 **Partial degradation by proteolysis**

193 In order to compare the accessibility of the variants to partial proteolysis,
194 proteins were incubated at 37°C in Tris buffer with Trypsin at a molar ratio apoA-I
195 variants to enzyme 1000:1. At different periods samples were heated in boiling water,
196 resolved by SDS PAGE, and developed by silver staining. The associated intensity of
197 the protein remaining within the monomer molecular weight was quantified with the
198 Image J 1.51 j8 Software. In the same trend, proteins were incubated with
199 metalloproteinase 12 (molar ratio protein to enzyme 500:1) and analyzed in the same
200 way.

201

202 **Detection of protein aggregates**

203 Thioflavin T (ThT) is widely used to detect amyloid-like structures [24].
204 Although ThT-associated quantum yield usually increases proportionally to the
205 formation of fibrillar protein aggregates, significant fluorescence is detected even when
206 proteins are present as oligomeric conformations [25][26]. To address the effect of pH
207 on the yield of amyloid-like protein aggregates, Wt (0.2 mg/mL) was taken for 48 h at
208 37°C at different pH between 7.4 and 5.0 in Citrate phosphate buffer, and ThT added at
209 a molar ratio of two with respect to protein. Associated fluorescence intensity was
210 measured on a Beckman Coulter DTX 880 Microplate Reader (Beckman, CA) through
211 the excitation (430 nm) and emission (480 nm) filters. The relative size of the
212 aggregates was estimated by light scattering on the SLM4800 spectrofluorometer
213 measuring light intensity at 90° with excitation and emission wavelength set at 400 nm.

214 Next, to compare the efficiency of the variants to bind to heparin, 0.2 mg/mL of Wt,
215 W50R, and L60R, were incubated for 48 h at 37°C, pH 5.0. Both fluorescence and
216 scattering were determined in the Microplate Reader, in the latter case by fixing
217 excitation and emission filters at 350 nm.

218 To characterize nanometer-scaled aggregates conformations, Transmission
219 electron microscopy (TEM), was performed on a JEOL-1200 EX. Negative staining is
220 widely used to increase the contrast as the stain surrounds the sample but is excluded
221 from the occupied volume and is thus observed as 'negative stain' [27]. Samples
222 incubated at 0.6 mg/mL (37°C for 7 days) were seeded on Formvard grids, 0.5%
223 phosphotungstic acid was added and visualized by negative staining. Magnification was
224 100,000 x.

225

226

227 **Lipid interaction properties of apoA-I variants**

228 Sodium dodecyl sulfate (SDS) is a negative lipid which mimics some
229 characteristics of biological membranes [28]; in addition, it has been described to elicit
230 the formation of fibrils from different peptides and proteins if used below the critical
231 micellar concentration (CMC). We have previously shown that Wt binds to this lipid
232 increasing ThT fluorescence [15]. To analyze the comparative binding of SDS to apoA-
233 I variants, proteins were incubated in citrate phosphate buffer pH 7.4 for 48 h at 37°C in
234 the presence or absence of 0.2 mM SDS. Previously we have confirmed that under those
235 conditions SDS is far lower from the CMC. ThT-associated fluorescence and scattering
236 were measured as described above.

237 DMPC clearance assay was used to give account for apoA-I function in lipid
238 solubilization. As proteins are incubated with this phospholipid at its transition

239 temperature the decrease in turbidity indicates the efficiency to form lipid-protein
240 complexes. DMPC was solubilized in chloroform and desired amounts were dried under
241 extensive N₂ flow and additional vacuum. Lipids were resuspended in Tris buffer and
242 Multilamellar liposomes (MLV) were obtained by exhaustive vortexing. Lipid clearance
243 was determined by incubating proteins with DMPC MLV at a molar ratio lipid:protein
244 40:1 at 24°C for 90 min and absorbance monitored on the Microplate Reader by setting
245 filters at 350 nm [15].

246

247 **Pro-inflammatory response induced by variants**

248 In order to determine whether a soluble conformation of W50R and L60R could
249 elicit cellular pro-inflammatory pathways, the human-derived THP-1 Cell Line (from
250 Leukemic monocytes, ECACC, Salisbury, UK) was seeded (10⁶ cells/mL) in RPMI
251 1640 medium in the presence of 10% fetal bovine serum (FBS), 100 U/mL Penicillin,
252 and 100 ug/mL Streptomycin at 37°C in a humidified incubator containing 5% CO₂.
253 Monocytes were activated by the addition of 5 ng/mL of Phorbol esters for 48 h [29].
254 Transformation into macrophages was observed by cellular adhesion to the plate.
255 Thereafter, medium was removed and apoA-I variants (at 1.0 µg/mL) added to the cells
256 and incubated for 3 h in RPMI medium plus 0.5% FBS, in the presence of the same
257 antibiotic mixture and Polymyxin B (final concentration 50 µg/mL). Positive and
258 negative controls were determined by addition of 50ng/mL of bacterial lipo
259 polysaccharide (LPS) in the absence of the presence of Polymyxin B, respectively. Cells
260 were spun at 2,100 rpm for 10 min to remove cellular debris, supernatant separated and
261 tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) release were compared by
262 specific enzyme immunoassay from BD Biosciences (San Diego, CA) used according to
263 the manufacturer's instructions. Cell viability under these incubation conditions was

264 checked by the 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazoliumbromide (MTT) cell
265 viability assay as previously described [14].

266

267 **Other analytical methods**

268 Protein content was quantified by the Bradford technique [30] or by absorbance
269 from the estimation of the extinction coefficient ($32,430 \text{ M}^{-1}\text{cm}^{-1}$ at 280 nm) as
270 determined in a Bio-Rad spectrophotometer (Hercules, CA). Unless otherwise stated, the
271 results were reproduced in three independent experiments and are indicated as means \pm
272 standard error. Statistically significant differences between experimental conditions
273 were evaluated by the Student's test.

274

275 **Results**

276 **Structural comparison and stability**

277 The expression and isolation procedures used in this study yielded high amounts
278 of pure proteins. We have previously shown that the lack of the two first amino acids
279 resulting from the acidic cleavage does not introduce modifications in protein function
280 or structure with respect to the plasma apoA-I [20]. In order to confirm the chemical
281 integrity and purity, the Wt variant was subjected to LC-MS/MS equipped with an
282 Orbitrap analyzer. Exponentially Modified Protein Abundance Index (emPAI) estimated
283 an abundance of 99.95 for this protein. Previous to each experiment, in order to ensure a
284 fresh folding, proteins were solubilized in GdmCl 2 M and extensively dialyzed through
285 the desired buffer.

286

287 Due to the location in the primary structure of the four Trp residues in apoA-I
288 molecule (8, 50, 72 and 108), the average intrinsic fluorescence is representative of the
289 conformation of the N-terminal domain (residues 1-184) [31]. In order to compare
290 proteins structure, we prepared freshly refolded apoA-I variants and characterized Trp
291 fluorescence spectra. While the wavelength of maximum fluorescence (WMF) of W50R
292 was similar to the Wt, a small but significant red shift (4 nm +/-1) was observed for
293 L60R (Table 1).

294 **Table 1 Spectral characterization of N terminal apoA-I variants.**

	WMF^a pH 7.4 (nm)	Relative intensity^b	K^c	AG^o(H₂O)^d (kcal/mol)	WMF^a pH 5.0 (nm)
WT	338 +/- 1	5.5 +/- 0.3	5.6 +/- 0.3	2.3 +/- 0.1	339 +/- 2
W50R	340 +/- 2	3.8 +/- 0.5	8.6 +/- 0.4	1.7 +/- 0.1	340 +/- 2
L60R	342 +/- 2 **	3.6 +/- 0.5	8.3 +/- 0.4	ND	342 +/- 2

295

296 ^a Wavelength of maximum fluorescence ** denotes difference with respect to Wt at
297 P<0.01.

298 ^b Fluorescence Intensity at the WMF, with all the proteins at 0.1 mg/mL and under
299 exactly the same slit and high voltage gain

300 ^c Stern-Volmer quenching constant for the quenching of 4 Trp residues by Acrylamide

301 ^d Free energy change of unfolding from equilibrium unfolding curves as shown in Fig 1.

302

303

304 Both mutants showed lower intensity than the Wt for identical protein
305 concentration. A significantly higher Stern-Volmer constant (K) calculated from the
306 analysis of Trp quenching with acrylamide (8.6 \pm 0.4 for W50R and 8.3 \pm 0.4 for
307 L60R) indicates a higher exposure of the Trp environments to solvent in both mutants
308 as compared to the Wt form (K 5.6 \pm 0.3).

309 To better estimate apoA-I natural variants' conformational stability, fluorescence
310 was followed after titration with GdmCl. The chemical denaturation pattern obtained
311 from the shift in the center of mass of the Trp emission has been extensively used to
312 give account for the protein stability under chaotropic solvents [32]. As previously
313 discussed, this parameter is in good agreement with the measurement of the emission
314 intensity at a fixed wavelength at increasing GdmCl concentration, and better help to
315 compare our previous data [14][15]. While the free energy of denaturation ($\Delta G^\circ_{H_2O}$)
316 for W50R (1.7 kcal/mol) is lower than the value estimated for the Wt (2.3 kcal/mol), the
317 dependence fit of Trp emission with GdmCl is far from a two state (native and
318 unfolded) model for L60R (Fig 1A), and thus we were not able to precisely calculate
319 this parameter. Interestingly, its total intrinsic fluorescence intensity is lower than the
320 one of the Wt, even though it keeps the four native Trp residues. As long as protein is
321 unfolded by titration with GdmCl the total intensity tends to equal that of the Wt (Inset
322 Fig 1A).

323

324 **Fig 1. Structural characterization of apoA-I variants.** Chemical equilibrium
325 unfolding of apoA-I in Tris 20 mM buffer pH 7.4 (A) and pH 5.0 in Citrate phosphate

326 buffer (B) was evaluated at a starting protein concentration of 0.2 mg/mL; Trp
327 fluorescence was registered with excitation at 295 nm and emission between 310 and
328 400 nm; spectral center of mass was plotted as a function of [GmdCl]. Circles, squares
329 and triangles represent Wt, W50R, and L60R, respectively. Lines correspond to fittings
330 of a sigmoideal model to the data. Y-left axe corresponds to Wt and W50R, and Y-right
331 axe to L60R. Inset Fig A represents the dependence of the total intensity of L60R
332 respect of Wt as a function of [GmdCl]. Circular dichroism in the far (C) or near (D)
333 UV region are represented for Wt (dark), W50R (dark grey) and L60R (clear grey).

334

335

336 In order to test whether a local decrease in pH could induce a disruption in
337 protein structure, variants were taken to pH 5.0 and denaturation curves repeated under
338 the same conditions. As Fig 1B shows, the behavior remains similar to that obtained
339 under physiological pH, indicating a mild effect of an acidic milieu on proteins`
340 conformation.

341 CD analysis of protein structure in the far UV reveals a conserved α helical
342 secondary structure which was previously identified for the Wt protein at low
343 concentrations (Fig 1C) [33]. The secondary structure content was calculated with the
344 algorithm CONTIN rendering a high percentage of helical structure for all the three
345 proteins [34]. The Wt spectrum is almost indistinguishable from that of W50R and
346 slightly more intense than that of L60R. Accordingly, a loss of about 4 and 7% of alpha
347 helical structure is observed for the W50R and L60R variants, respectively.

348 The comparison among the tertiary structures was analyzed by CD in the near
349 UV (Fig 1D); spectra of both mutants preserve fine structure although the lower

350 intensity from 280 nm to 250 nm indicates mild structural differences among the
351 aromatic amino acid residues.

352 Partial proteolysis sensitivity is usually evaluated in order to give information
353 about the accessibility of protein domains within the spatial arrangement. Thus, variants
354 were incubated at increasing times with either trypsin or metalloproteinase 12
355 (MMP12), and run through an SDS PAGE visualized with Silver staining; the time
356 dependent efficiency of the proteolysis could be observed and quantified by the
357 disappearance of the band corresponding to the original Molecular weight (Fig 2A). The
358 digestion rate of the major fragment is higher as long as the incubation time increases
359 indicating the efficiency of the proteolysis.

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361

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363 **Fig 2. Partial proteolysis of apoA-I variants.** Proteins were incubated at 0.3 mg/mL in
364 Tris 20 mM buffer pH 7.4 with trypsin or MMP12 at molar ratios of 1000:1 or
365 500:1apoA-I variants to enzyme respectively. After different time periods, reactions
366 were stopped by the addition of sample running buffer and two minutes boiling.
367 Samples were run through a SDS PAGE (16%) and developed by silver staining. A)
368 Black/white representation of the initial (0) and final (60 min) incubation times.
369 Intensity remaining with the monomeric molecular weight (28 kDa) after B) Trypsin or
370 C) MMP12 treatments was quantified by the Image Quant software and normalized to
371 the intensity of the band at time= 0 for each protein. Circles, squares and triangles
372 correspond to Wt, W50R and L60R respectively (** represents $P < 0.001$ with respect to
373 the same time in the Wt).

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377 After 45 minutes incubation under these conditions and in agreement with Das et
378 al [16] the proteolysis of W50R showed a similar yield as the Wt, instead it
379 demonstrated to be significantly more efficient for L60R with both enzymes tested.

380 The extrinsic fluorescence probe ANS (or its dimer Bis-ANS) was extensively
381 used to test the spatial arrangements of proteins, due to the fact that is it weakly
382 fluorescent in water but its quantum yield increases significantly, and its emission shifts
383 upon binding to proteins. Its emission is proposed to sense specifically protein
384 hydrophobic pockets and “molten globule” like states [32]. We and others have
385 previously shown that binding of Bis-ANS to apoA-I Wt is efficient and fast [14][15].
386 We herein show (Fig 3), as it was previously demonstrated with the monomer ANS, that
387 W50R binds Bis-ANS with a relatively higher quantum yield than the Wt [17].

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391 **Fig 3. Binding of Bis-ANS to apoA-I variants** Proteins were diluted at a final
392 concentration of 0.1 mg/mL (Tris 20 mM pH 7.4 buffer) and titrated with small
393 amounts of Bis-ANS to a final concentration of 16 μ M. Fluorescence was detected in
394 the SLM 4800 spectrofluorometer setting excitation at 360 nm, and intensity of the
395 emission registered at the observed Wavelength of Maximum Fluorescence (WMF) for
396 the probe (490 nm). As in Fig. 1 circles, squares and triangles represent Wt, W50R, and
397 L60R, respectively. Inset: spectra of Bis-ANS at a molar ratio 4:1 probe to protein
398 normalized at the WMF. Continuous and dashed lines represent Wt and L60R
399 respectively.

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403 Instead, intensity of the Bis-ANS associated to L60R is lower indicating a lower
404 permeation or binding of this probe within the spatial protein arrangement.
405 Nevertheless, WMF of the Bis-ANS bound to L60R is similar to the probe associated to
406 the Wt (Fig 3 inset). In a different experiment, to evaluate whether the presence of the
407 hydrophilic group in the mutants may disrupt the protein self-association, freshly folded
408 Wt and variants were run under FLPC; the elution pattern demonstrated that most of the
409 Wt migrates as a dimer with a minor fraction (about 20%) eluting as a monomer (S. Fig
410 1). This pattern is well preserved for both mutants.

411

412

413 **Aggregation tendency**

414 The variants tendency to aggregate under physiological pH and low
415 concentrations was evaluated by incubating proteins at 0.2 mg/mL for 48 h in Citrate
416 buffer pH 7.4. The ThT measurement indicates as previously observed for the Wt that
417 amyloid complexes were not detected under these conditions (dark gray bars in S. Fig
418 2). In a parallel experiment, proteins were further incubated at 0.6 mg/mL for 7 days
419 under the same conditions and observed by Transmission Electron Microscopy (Fig 4).

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424 **Fig 4. Observation of apoA-I mutants' aggregates at physiological pH.** Wt (A),
425 W50R (B) or L60R (C) were incubated for 7 days at 0.6 mg/mL and 37 °C in Citrate
426 phosphate buffer pH 7.4. Aggregates were observed by negative staining under
427 Transmission Electron Microscopy on a JEOL-1200 EX Microscope. Bars in each
428 image show the scale used.

429

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431

432 The sample of the Wt showed a low degree of amorphous-like aggregates and
433 small amounts of protofibrils (Fig 4A). Both mutants (W50R and L60R, Fig 4B and C
434 respectively) were represented by a higher yield of aggregates with similar morphology.
435 In addition protofibrils of about 8-10 nm diameter are observed under these conditions.
436 The behavior of these proteins was similar to other mutants analyzed under the same
437 conditions [14][15].

438

439 **Binding to ligands**

440 Heparin is usually used as a model of glycosamino glycans (GAGs), which are
441 supposed to play key roles in the maintenance of cellular functions. Moreover, it is
442 proposed that interactions of proteins with GAGs could result in the retention or
443 aggregation of proteins inducing amyloidosis [35][36], but instead they could compete
444 avoiding cellular toxicity or conformational shifts [37]. Within this frame in mind, we
445 analyzed binding of both natural mutants to heparin either at physiological pH or under
446 acidic conditions. ThT results suggest, as observed before for the Wt under these
447 conditions [19], that the presence of heparin does not induce a significant structural
448 arrangement of the variants when incubated at pH 7.4 for 2 days at 37°C (clear gray bars

449 in S Fig 2). As long as pH decreases, protonation of His residues may result in the gain
450 of positive charges of the proteins thus helping electrostatic interactions with the
451 negative groups of the GAGs. As the isoelectric point of apoA-I is estimated in 5.27, we
452 analyzed the effect of an acidic environment on apoA-I aggregation and interaction with
453 heparin by incubating Wt at different pH between 5.0 and 7.4, in the absence or the
454 presence of this model of GAG. Size and amyloid-like tendency of the complexes were
455 followed by scattering (Fig 5A) and ThT fluorescence (Fig 5B). These data indicate that
456 the formation of Wt amyloid-like complexes is favored as long as pH decreases (dark
457 gray bars), and this tendency is increased in the presence of heparin (clear gray bars).

458

459 **Fig 5. Influence of acidic pH on heparin binding.** A and B) Wt was solubilized at 0.2
460 mg/mL at different pH from 7.4 to 5.0 by using Citrate phosphate buffer and incubated
461 either in the absence (dark gray bars) or the presence (clear gray bars) of heparin at a
462 molar ratio 1:1 protein to GAG for 48 h at 37°C. A) Light scattering at 90° was
463 determined in the spectrofluorometer with excitation and emission wavelengths at 400
464 nm; B) ThT associated fluorescence was determined in the Multiplate Reader with
465 excitation set at 430 and emission filter at 480 nm. Bars correspond to mean \pm SD.
466 Differences were analyzed by ANOVA followed by Tukey test. Different letters
467 symbolize significant differences ($P < 0.05$). C and D) Wt, W50R, and L60R, were taken
468 at pH 5.0 in Citrate phosphate buffer (0.2 mg/mL) and incubated in the absence (dark
469 gray bars) or the presence (clear gray bars) of heparin at a molar ratio of 2 per mol of
470 protein at 37°C by 48 h. C) Scattering was analyzed in the Microplate Reader with filter
471 set a 340 nm; D) ThT associated fluorescence was quantified as described in Fig 5B.
472 Symbol ** in C and D corresponds to differences with $P < 0.001$ with respect to Wt
473 under the same conditions.

474

475

476 Next, we compared the binding of the variants under study to this model of GAG
477 at pH 5.0. As shown, a higher scattering (Fig 5C) and ThT associated fluorescence (Fig
478 5D) in the case of W50R indicates its higher efficiency to bind to heparin as amyloid-
479 like complexes under acid conditions while L60R behaves similar to Wt.

480 In order to answer whether the structural disruption induced by a change in the
481 amino acid sequence (and charge) of the apoA-I variants could modify their interactions
482 with negative ligands in the microenvironment, we incubated W50R and L60R with
483 SDS, which not only is a good model of membrane lipids but also was suggested to
484 work as inductor of amyloid-like complexes formation from different proteins when
485 incubated under the lipid critical micellar concentration (CMC: 0.7 mM) [38]. Fig 6
486 shows, as previously observed, a strong increase in ThT fluorescence of Wt protein
487 when bound to SDS [15] which is in a similar trend for W50R and with a higher yield
488 for L60R.

489

490

491

492 **Fig 6. Protein binding to SDS.** ApoA-I were incubated at a concentration of 0.2
493 mg/mL in Citrate phosphate buffer pH 7.4 in the absence (dark gray bars) or in the
494 presence (clear gray bars) of 0.2 mM SDS. After 48 h at 37°C ThT was added to a 1:1
495 molar ratio to protein and relative fluorescence quantified as described in Figure 5D.
496 The symbol * denotes difference with respect to Wt at P<0.05.

497

498

499 **Effect of mutations on lipid clearance and cellular responses**

500 It is clear that the conformational flexibility could alter the equilibrium between
501 function and toxicity, and thus we set out to test whether the structural shift detected in
502 the N terminus of L60R could affect the protein interaction with a model of membrane.
503 It is well known that the analysis of MLV clearance at the lipid transition temperature is
504 a traditional functional parameter to test efficiency of apoA-I for lipids solubilization.
505 Fig 7 shows that the L60R mutant clears DMPC MLV with a small but significantly
506 higher efficiency than the Wt indicating a conserved function in lipid binding.

507

508

509

510 **Fig 7. Characterization of the mutant L60R on the spontaneous formation of**
511 **lipid:protein complexes.** Multilamellar DMPC liposomes were incubated at 24°C in
512 the presence of Wt (circles) or L60R (triangles) at a 80:1 lipid to protein molar ratio.
513 Absorbance was measured at 340 nm in the Microplate Reader. Significant difference
514 among both variants was determined by comparing absorbance at the last point of the
515 measured kinetics (120 min). L60R Absorbance was different with respect to Wt at
516 $P < 0.05$ as measured by the Student's Test.

517

518 Finally, and in order to test the hypothesis that apoA-I variants could induce
519 cellular responses associated to a pro-inflammatory landscape, we compared the release
520 of TNF- α and IL-1 β from THP-1 cells, which is widely used as a human model of
521 macrophages. As Fig 8A and B show, while W50R behaves as the Wt, L60R induced
522 the release of these cytokines. MTT reduction measurements indicated that cell viability
523 was preserved under the different conditions tested (not shown)

524

525

526

527 **Fig 8. Induction of TNF- α (A) and IL-1 β (B) release from cultured macrophages.**

528 THP-1 human monocytes were activated to macrophages by the addition of 5 ng/mL of

529 Phorbol esters for 48 h. One μ g/mL (A) or 0.5 μ g/mL (B) of Wt, W50 or L60R were

530 incubated with the cells for 3 h in the presence of Polymyxin B. Incubation of cells with

531 LPS, either in the presence (LPS +P) or absence (LPS -P) of Polymyxin B, was used as

532 negative and positive control respectively. P represents an extra negative control in

533 which cells are incubated only in the presence of Polymyxin B. Symbol #represents

534 significant difference respect to negative control (LPS +P) at $P < 0.05$. Symbol **

535 represents significant difference with respect to Wt at $P < 0.001$.

536

537

538 **Discussion**

539 Structural flexibility is essential for apolipoproteins in order to fulfill complex

540 functions, as it is required for apoA-I to interact within micro environmental ligands to

541 solubilize lipids; nevertheless, this property may put the proteins into the risk of

542 suffering subtle structural shifts from the native structure. The late-onset of the clinical

543 manifestations of the amyloidosis disease due to apoA-I variants supports the fact that

544 the final fibrillar conformation detected in the lesions is probably the result of

545 progressive events that cooperate to give rise to the insoluble protein aggregates. Thus,

546 it could be possible that a higher yield of partially folded variants could work late as

547 seeding events in the long term period.

548 The structural modifications that result from the natural mutations in apoA-I are

549 not easily predicted. Interestingly, although G26R, W50R, and L60R experiment a
550 similar substitution in the N terminus (the replacement of a neutral amino acid by an
551 Arg), only little disorder is observed for G26R [16] and W50R (this work and others
552 ([16]; instead the structural arrangement observed herein for L60R is more evident. Das
553 et al suggested that more than a strong conformational shift, the mutations in apoA-I
554 may induce perturbations, increasing the accessibility of amyloid-prone segments that
555 favor protein aggregation [16]. Such mild perturbations could increase the cleft from the
556 α helical bundles allowing the accessibility to not-yet-described proteases which help
557 the release of the peptides identified in the lesions. By an elegant model, they propose
558 that destabilization of the four-helix bundle containing residues may favor the
559 formation of a resistant amyloid core by interactions of the N-terminal amyloid hot
560 spots.

561 In agreement with Das et al. [16], our fluorescence and dichroism spectroscopic
562 measurements (a small red shift in the Trp environment and a preserved secondary
563 structure) suggest a mild effect of the Trp substitution by an Arg in position
564 50. Mutation W50R is comprised in the middle of the segment L44–S55 which was
565 shown in the crystal structure having an extended conformation consistent with the β -
566 strand-like geometry (Fig 9). It was proposed that the exposure of this segment could
567 initiate the α -helix to β -sheet apoA-I conversion in amyloidosis. Nevertheless, the
568 disruptive presence of a positive charge in replacement of the aromatic Trp is detected
569 in our experimental design by an increased yield in the Bis-ANS binding and minor
570 change in the near UV spectra, together with the detection of stronger binding to
571 heparin at acidic pH. It is worth mentioning that the N-terminal 1–83 fragment of W50R
572 variant was shown to participate in heparin-mediated fibril formation [39].

573

574

575 **Fig 9: Locations of amyloidogenic mutations W50R and L60R in the structure of**
576 **apoA-I.** The structure was obtained by the PyMOL Molecular Graphics System,
577 Version 2.0 (Schrödinger, LLC), from the X-ray crystal structure of $\Delta(185-243)$ apoA-I
578 (PDB ID 3R2P). N terminal residues subjected to mutations are shown in stick
579 representation.

580

581 Acidic intracellular milieu was associated to macrophages activation within
582 inflammatory lesions [40]. As mentioned above, the W50R variant is one of the few
583 exceptions in which apoA-I mutants' deposits are associated to the glomeruli [13][41].
584 The extracellular matrix (ECM), especially the proteoglycans (PGs) have diverse
585 biologic functions, including binding of growth factors and regulation of collagen
586 fibrillogenesis [42], and their composition is tissue specific [43]. In addition, it was
587 found that decorin (a dermatan/chondroitin sulfate PG) accumulated in amyloid
588 deposits, but not in deposits of fibrillary glomerulo nephritis [44]. From our previous
589 studies we learnt that binding of proteins to GAGs seems to be a cooperative, specific
590 interaction [45]. We have previously observed that the amyloidogenic mutant R173P
591 shows sensitive binding to heparin under physiological pH in spite of the loss in one
592 positive charge, probably due to the exposition of cryptic positive residues which are
593 accessible by the break in the amphipatic helix induced by the Proline residue in
594 position 173 [15]. Even though binding at physiologic pH is not clear for the mutants
595 tested here it is expected that the acidic milieu may strength protein-GAGs interactions.
596 Although further studies are worth to be done, it could be speculated that the positive
597 Arg charge could induce a stronger retention within the glomeruli's GAGs thus helping

598 its aggregation, especially under situations of kidney disease associated to
599 inflammation.

600 As mentioned above, L60R shows (as compared to Wt), a red shift in Trp
601 fluorescence which may indicate a relatively higher exposure of these aromatic residues
602 to the polar environment. Due to the average contribution of the 4 Trp in the native
603 structure, this shift may indicate either a subtle movement of side chains or larger scale
604 conformational changes of the protein. The observed lower binding to Bis-ANS may
605 suggest that the disruption of the bottom hydrophobic cluster by the positive charge of
606 the Arg (Fig 9) could bring this amino acid more exposed, as it results from a higher
607 binding to SDS which is not detected for the other amyloidogenic mutant tested in this
608 work (Fig 6); moreover, this structural disorder should allow the permeation of
609 proteases, as it is shown here. Although trypsin is not a physiological protease of apoA-
610 I in circulation, it helps to get insight into protein structure. In order to compare trypsin
611 induced proteolysis, we analyzed by the expasy software (www.expasy.org) the
612 predicted sites to be substrate of this enzyme. Arg in position 60 should separate only
613 one amino acid from a predicted fragment of three residues (59-61). Nevertheless, a
614 higher efficiency of this variant's processing is observed (Fig 2); the same tendency is
615 detected under this experimental design by using MMP-12. Altogether, the
616 rearrangement of the Trp environment in the L60R mutant, the higher accessibility to
617 proteases and the lower CD spectral signal at 255-280 nm respect to the Wt agree with a
618 spatial rearrangement with a decreased stability and an increase in protein flexibility.
619 The increased susceptibility to proteases could explain the appearance of a 10 kDa
620 molecular mass N terminal peptide within the lesions [8], although interestingly *in vitro*
621 studies with the 93-residue N-terminal fragment demonstrated that the peptide freed

622 behaves similar to Wt in the aggregation pattern than the same peptide with the Wt
623 sequence [46].

624 In addition to protein misfolding, it is worth to consider the possibility that at
625 least part of the clinical manifestations could be due to a loss in protein function, such
626 as lipid binding, which in addition could increase the amount of lipid-free protein,
627 amyloid-prone precursors. In order to consider this possibility, we first modeled the
628 domain in which the mutation occurs (amino acids 52-65) in a helix wheel modeling
629 software (<http://lbqp.unb.br/NetWheels/>). The modeling predicts that due to the
630 periodicity that brings in close proximity residues Val53, Leu64 and Phe57, the Leu 60
631 contributes to form the apolar phase of the putative amphipatic class A α -helix (Fig10).

632

633

634 **Fig 10. Helical wheel model of the putative α -helix comprising residues 52-65 of**
635 **apoA-I** Wt sequence represented as an helix seen down the long axis, with an amino
636 acid arrangement considering an ideal α -helix (100° rotation per amino acid). Gray scale
637 in the Figure corresponds to the nature of different amino acids. Toward the right side
638 of the helix it is indicated the replacement of Arg in position 60 in the nonpolar phase.

639

640

641 The positive charge of the Arg may thus perturb the hydrophobic bottom cluster.
642 The mutation in position 60 should in addition disrupt a Leu zipper stabilized with other
643 Leu residues in the same molecule or with a second molecule in the native dimer [47].
644 In our hands the shift among dimer and monomer conformation was not dramatically
645 modified (S. Fig 1). Moreover, this effect is not drastic enough to decrease the
646 efficiency to solubilize neutral lipids (Fig 7) as it is the case with W50R and G26R

647 substitutions [16]. Instead, it could be that the relative exposition of the positive charge
648 in this segment could favor its interactions and/or shift toward a pathological amyloid-
649 like structure when interacting with other ligands, as negative lipids, as it suggested by
650 the SDS binding experiments.

651 Finally, we have recently shown that other N terminal apoA-I mutant
652 (Gly26Arg), induces the release of TNF- α and IL-1 β from a model of macrophages
653 [14], in a pathway probably involving the specific activation of the NF- κ B pro-
654 inflammatory cascade [48]. The kidney is a major target organ of innate immune
655 inflammatory diseases. The deposition of the acute-phase reactant serum amyloid A
656 (SAA) as amyloid causes progressive glomerular and vascular damage and leads to
657 organ failure [49]. It was suggested that this and other misfolded proteins could be
658 recognized by pattern recognition receptors (PRRs) resulting in the activation of pro
659 inflammatory cascades, being increased IL-1 β secretion responsible for most of the
660 systemic features of this group of disorders [50]. IL-1 β production may induce the
661 synthesis of other cytokines as TNF- α . Our results shown here indicate that mild
662 conformational rearrangement detected for L60R variant may induce the activation of
663 the microenvironment toward a pro-inflammatory landscape which could help to
664 perpetuate events triggering organ damage.

665 In conclusion, in agreement with other groups, our data support that it is not
666 required a large overall destabilization of the tertiary structure of apoA-I to become
667 amyloidogenic; either reduced protection of the major amyloid “hot spot”, increased
668 susceptibilities to proteases or partial oxidation could induce cooperative shift toward a
669 misfolded conformation. Nevertheless, the chronic clinical phenotype indicates that the
670 landscape in which protein circulates (molecular crowding, acidification, oxidation,
671 interactions with ligands etc) could play key roles to give rise to amyloid species. These

672 species could either be nucleus of aggregation or, as we have previously suggested,
673 work as signal receptors starting up cellular events associated to the pathology. Further
674 research will help to explore different pathways.

675

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677

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682

683

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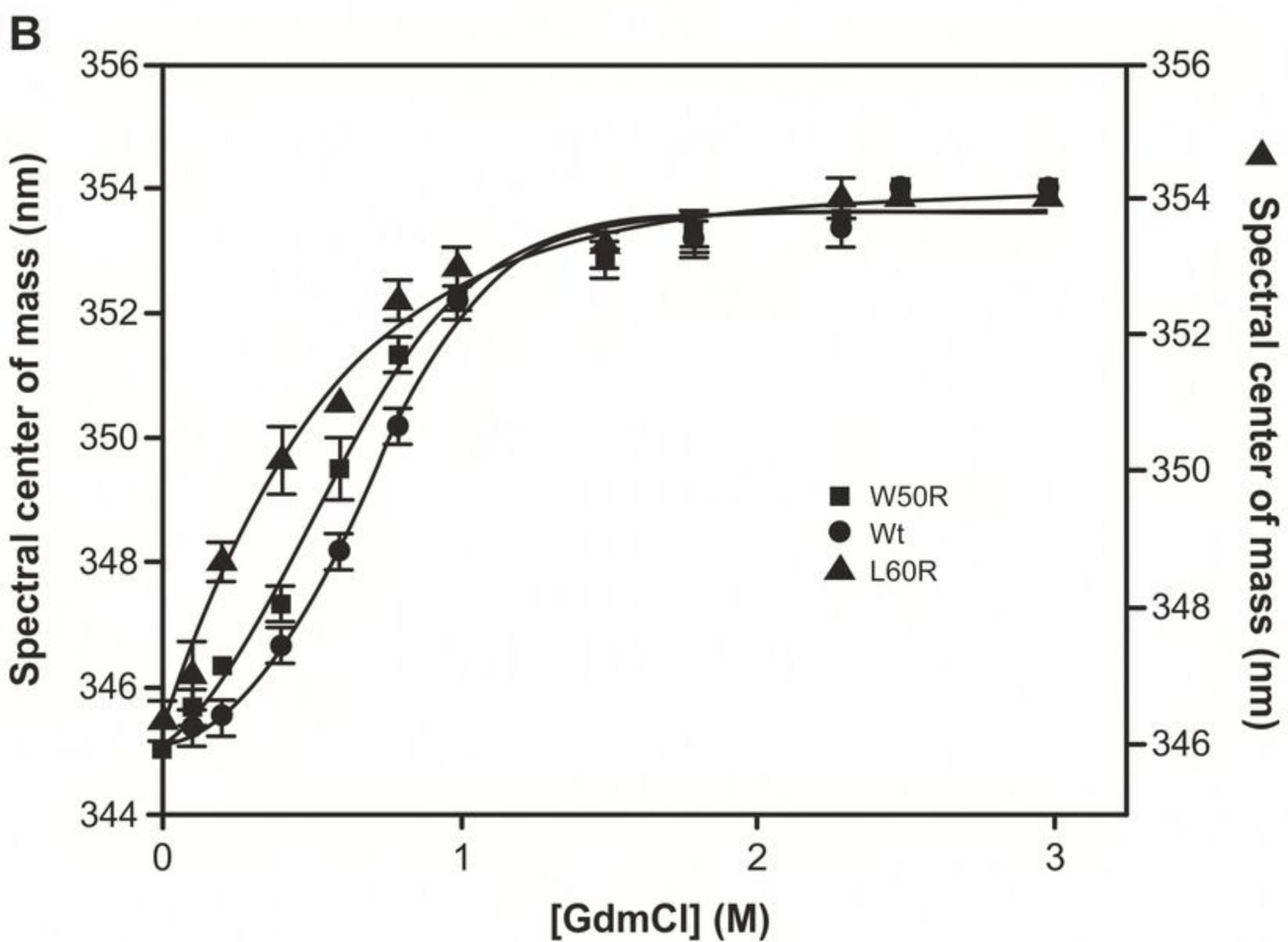
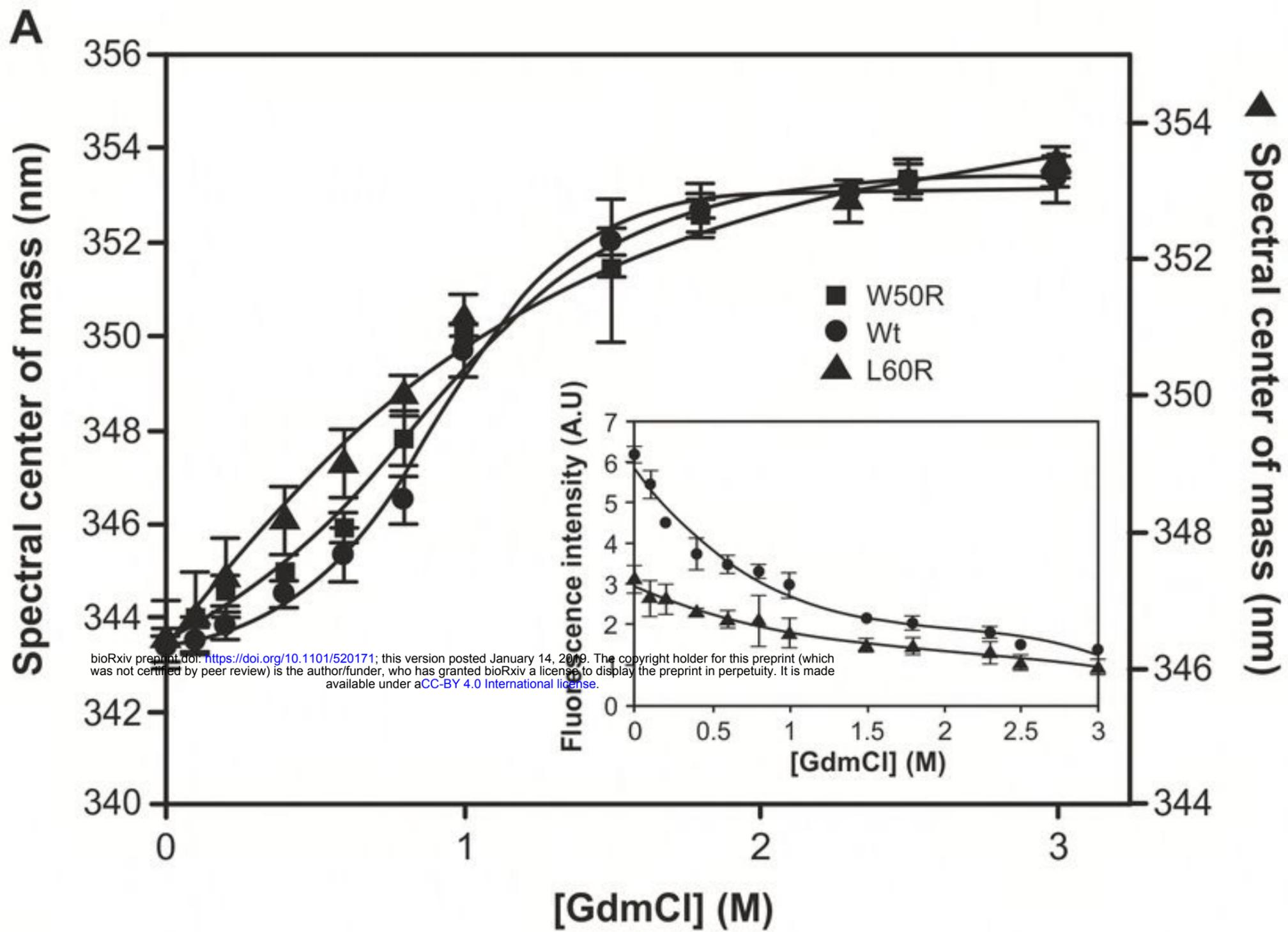


Figure 1 a-b

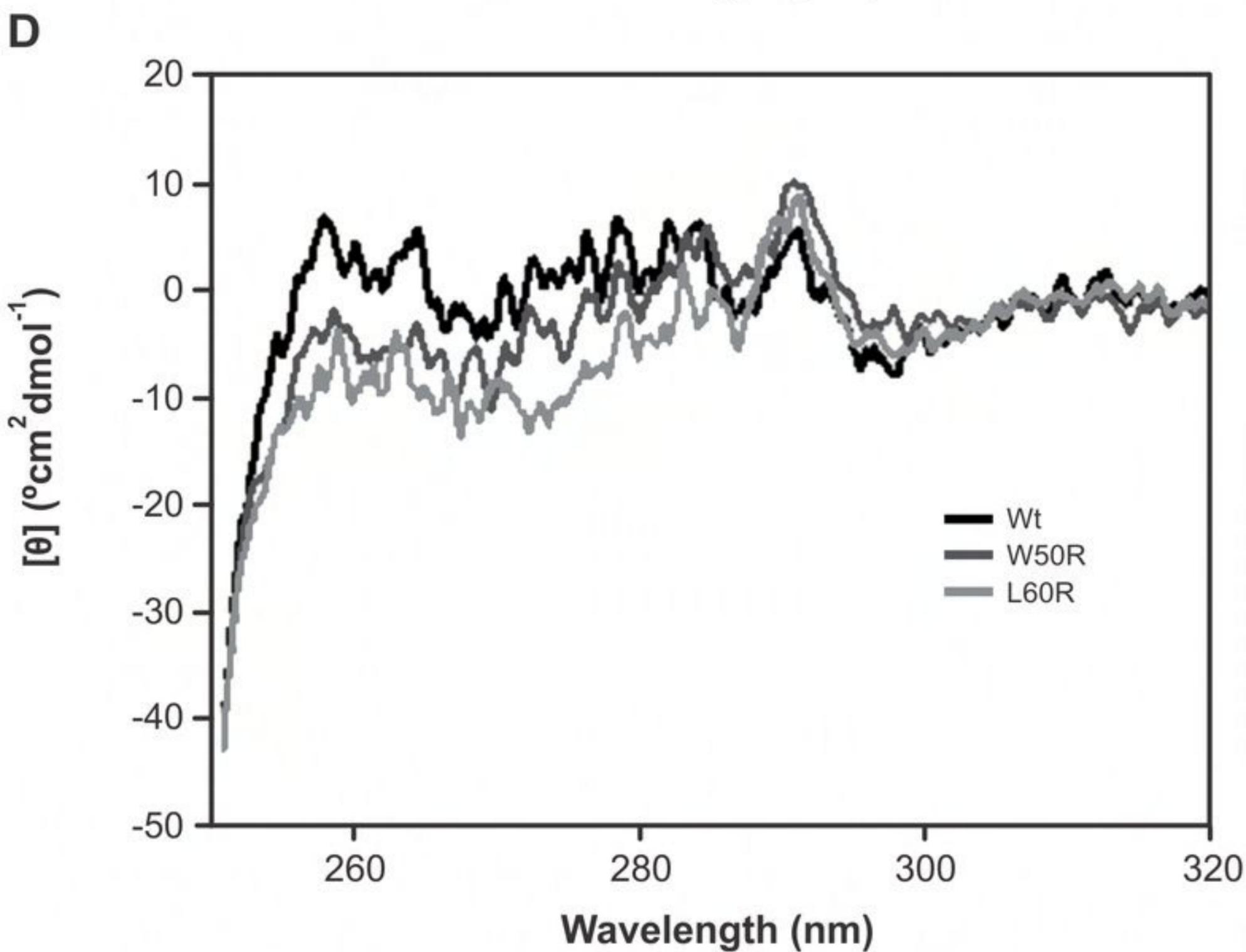
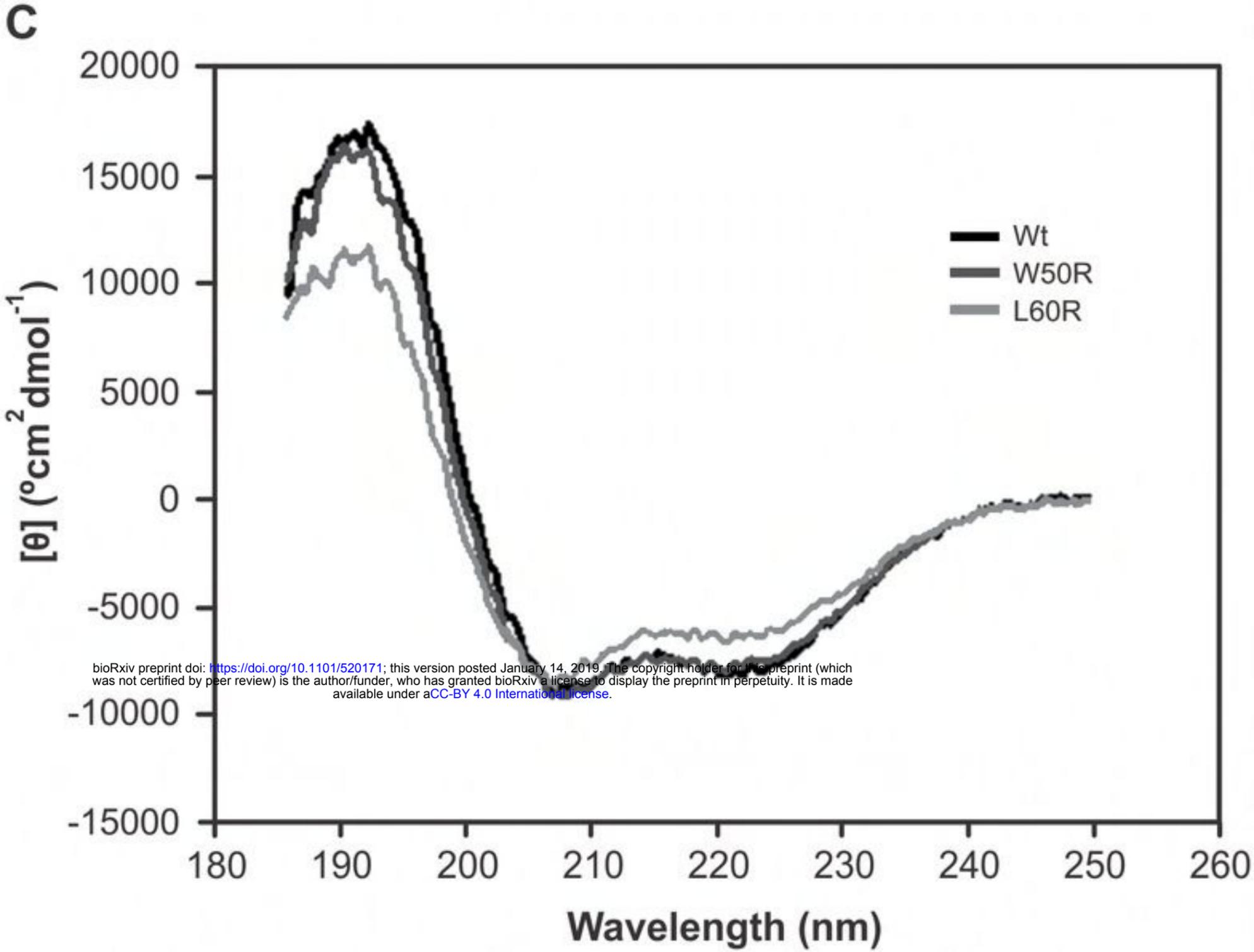


Figure 1 c-d

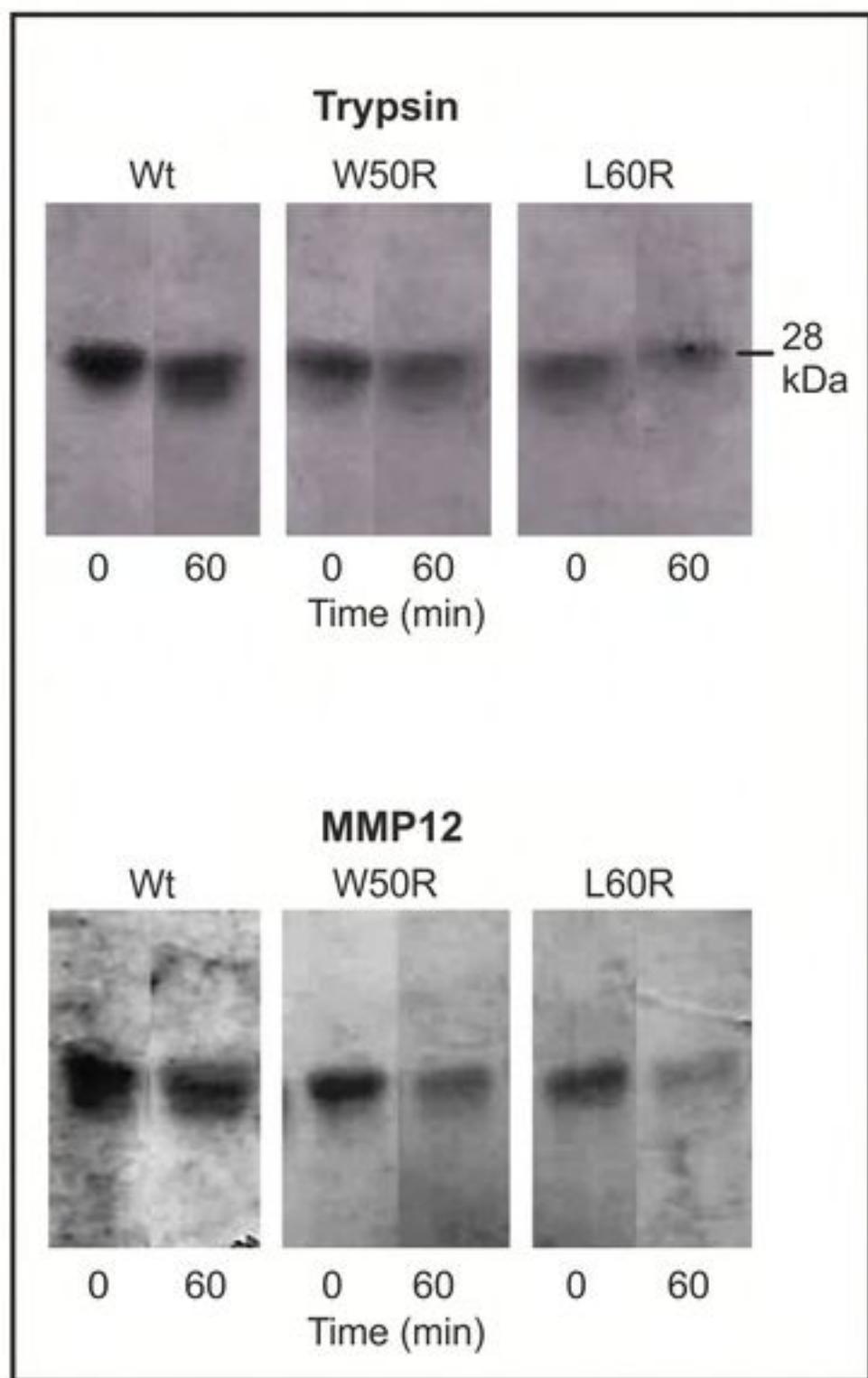
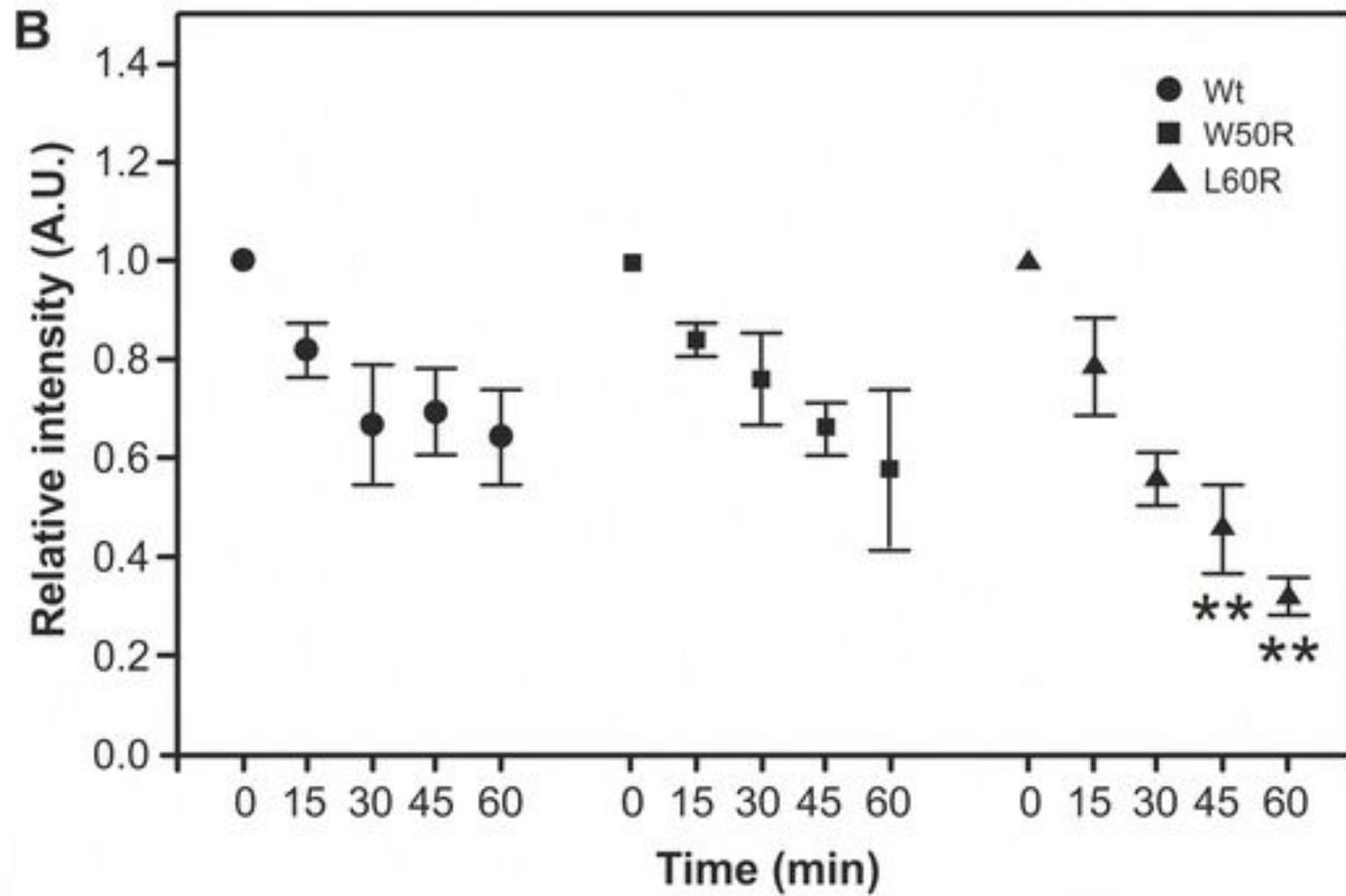
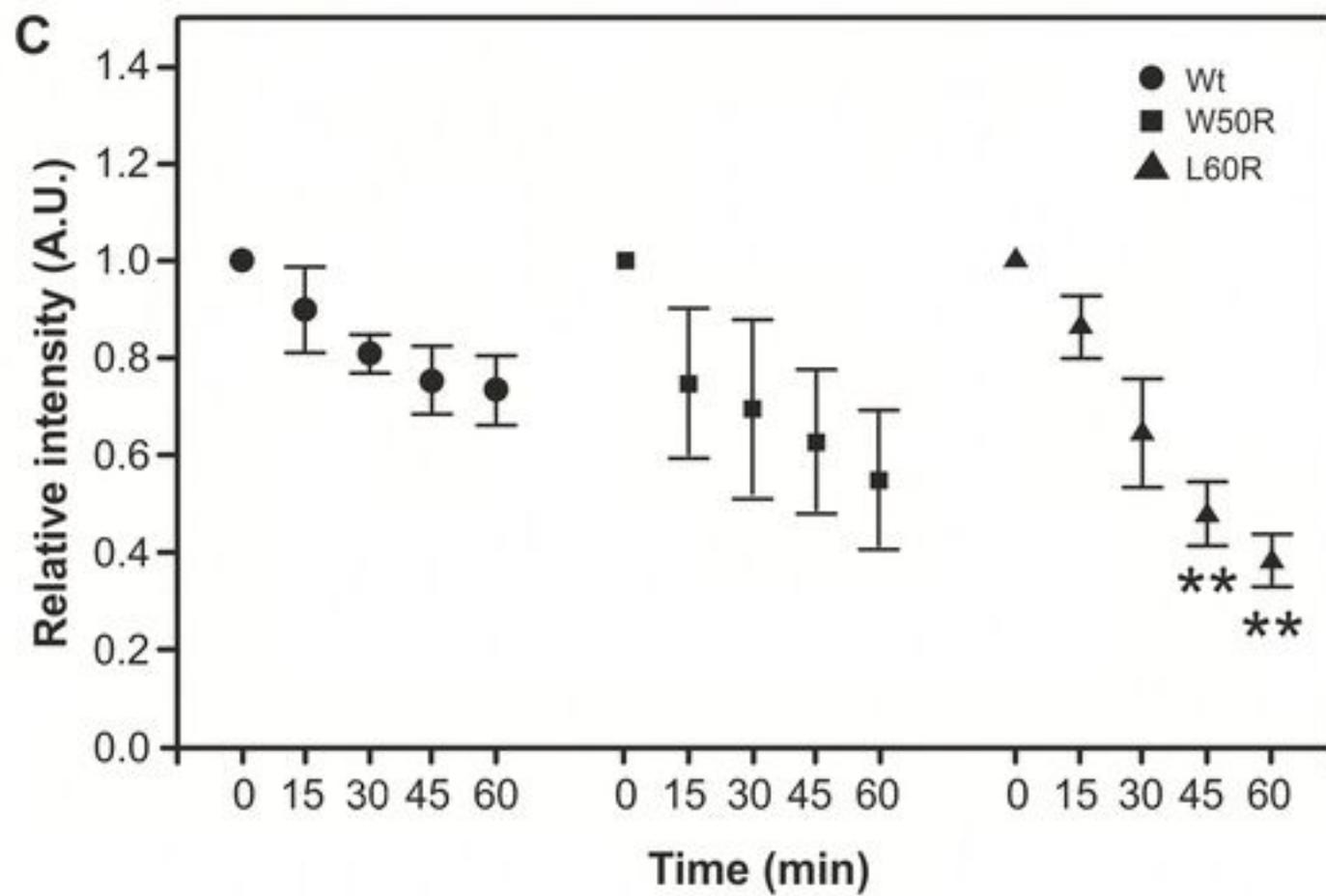
A**B****C**

Figure 2

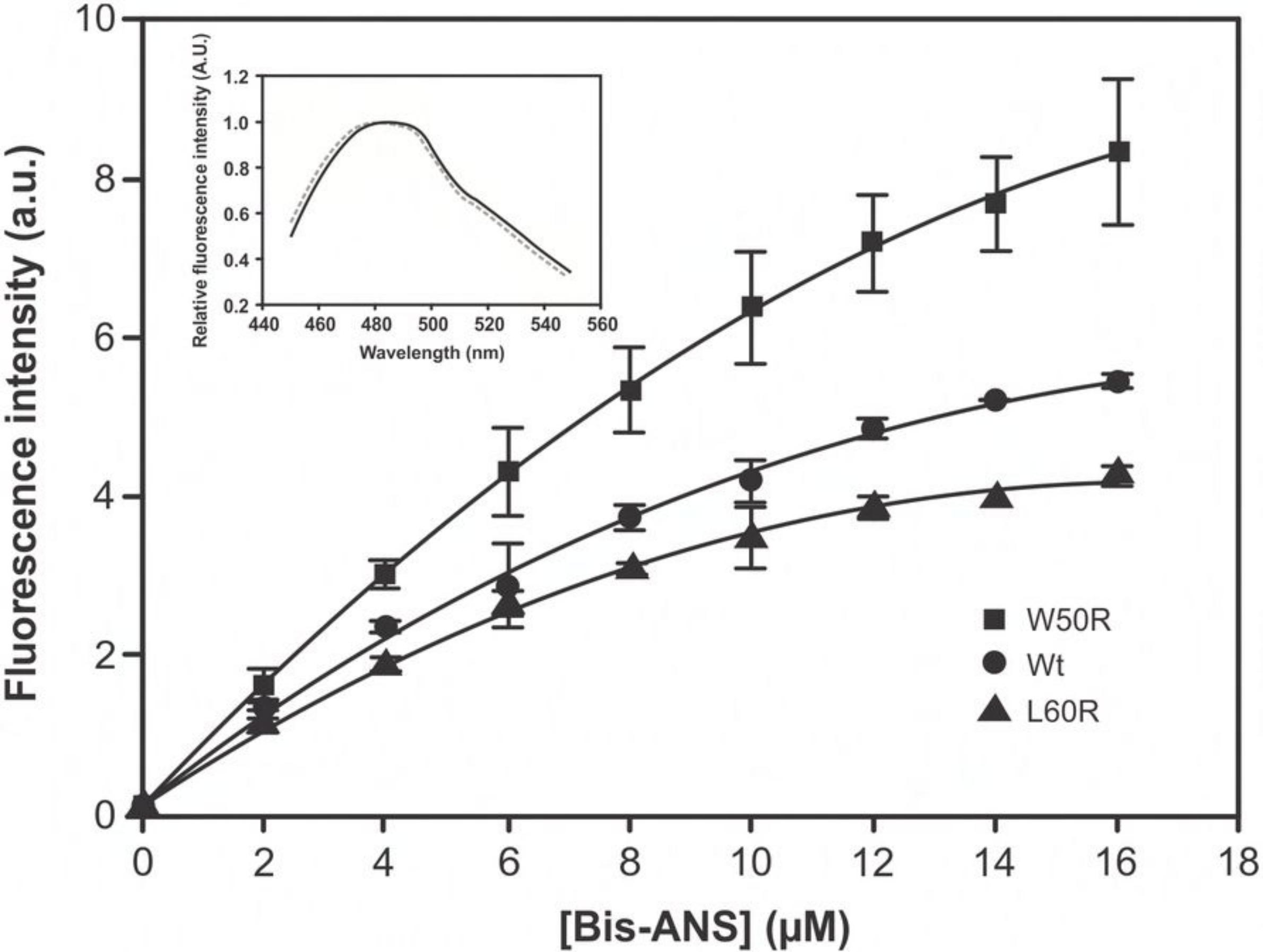


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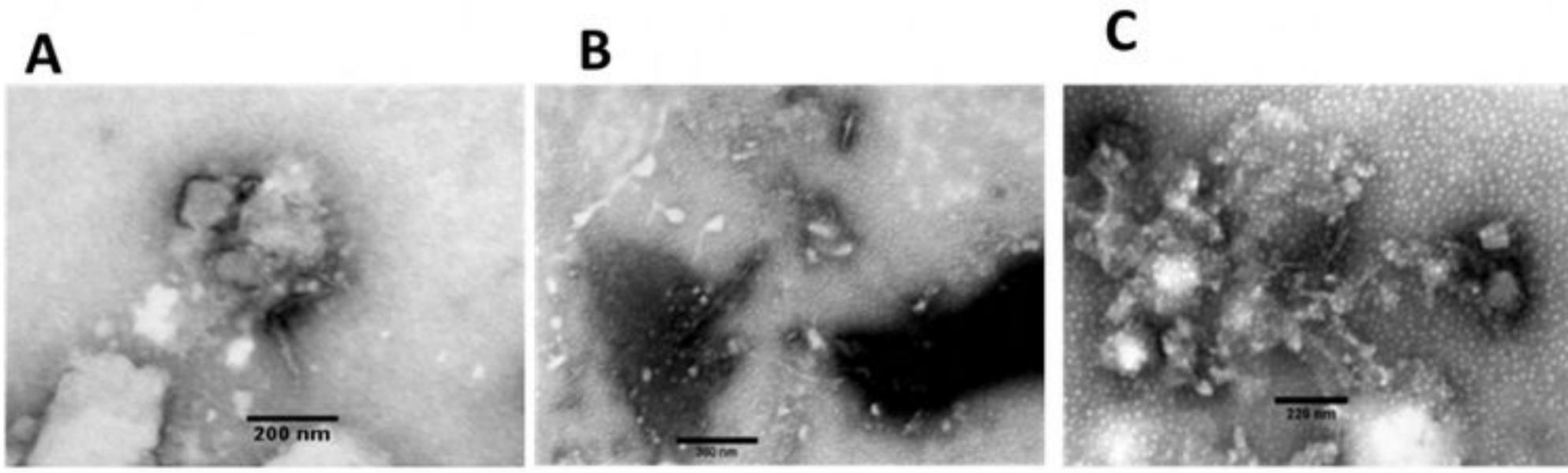


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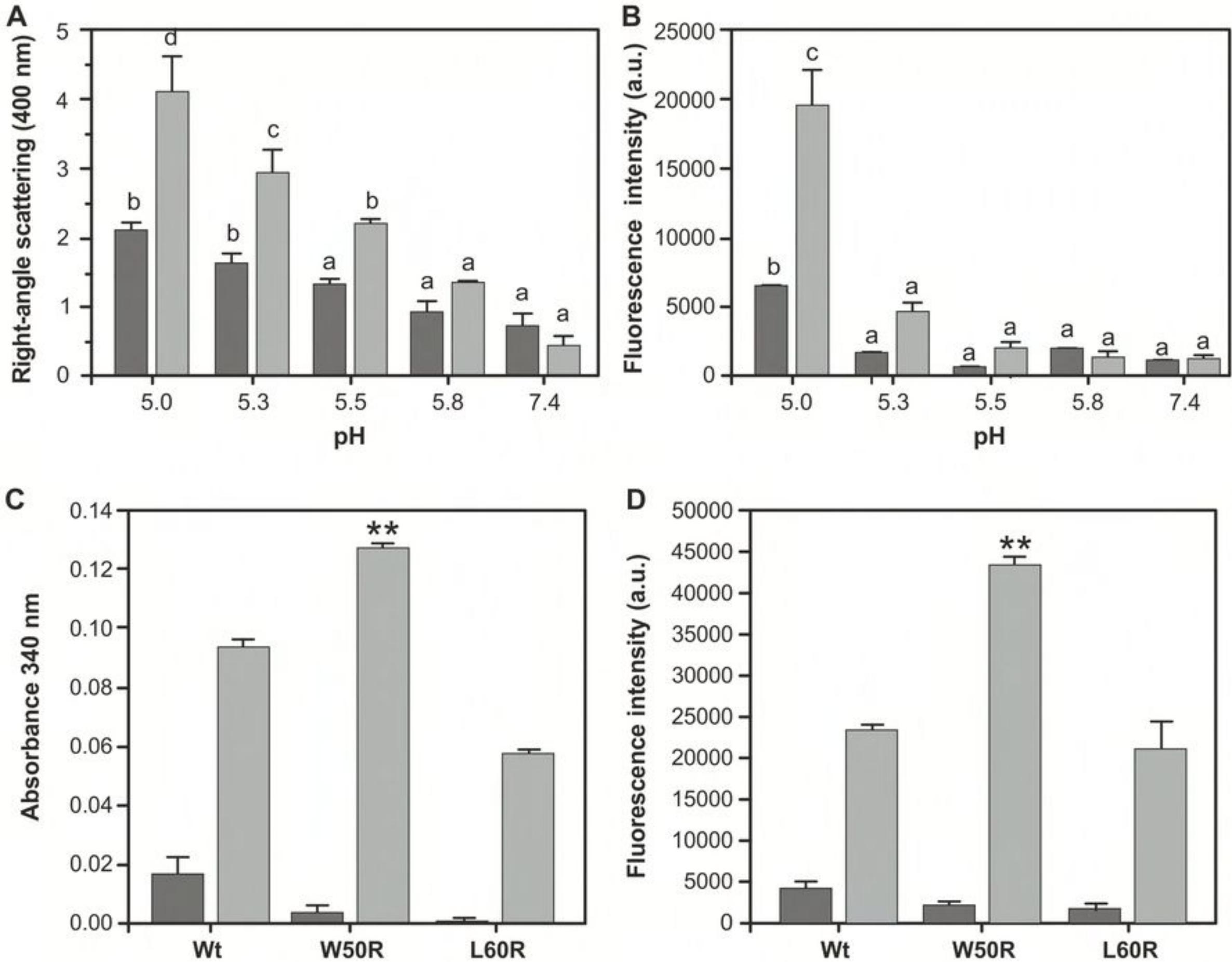


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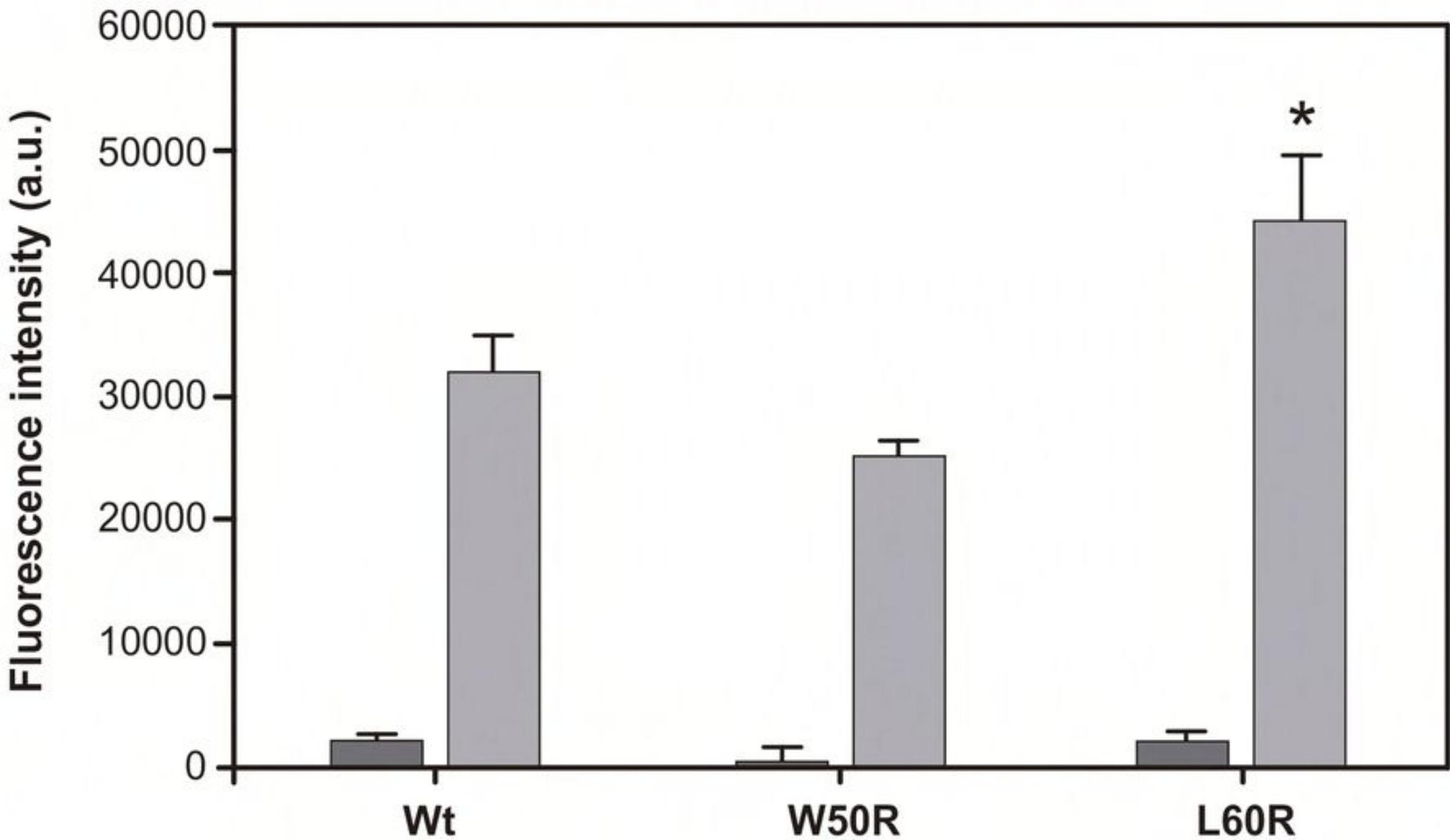


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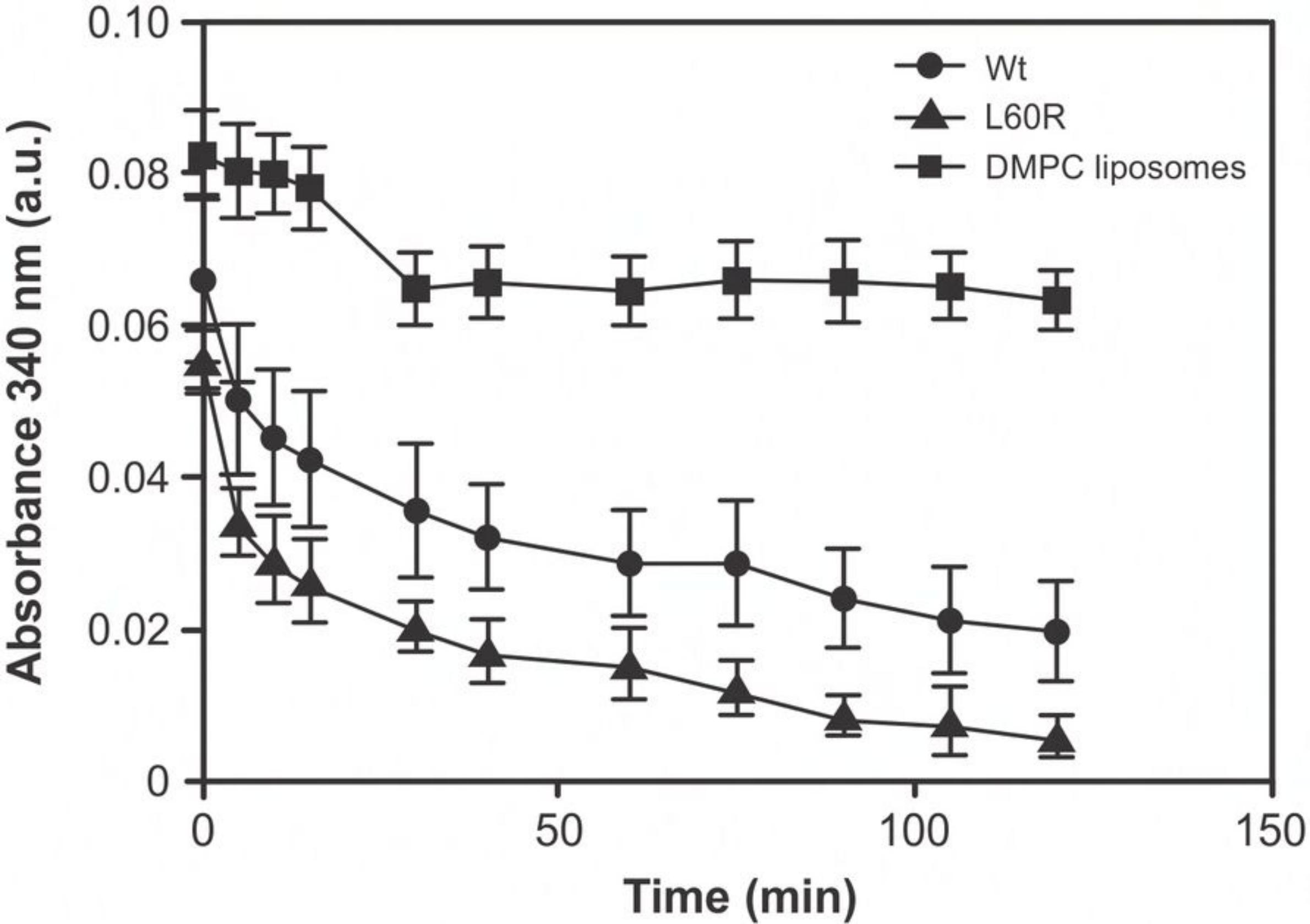


Figure 7

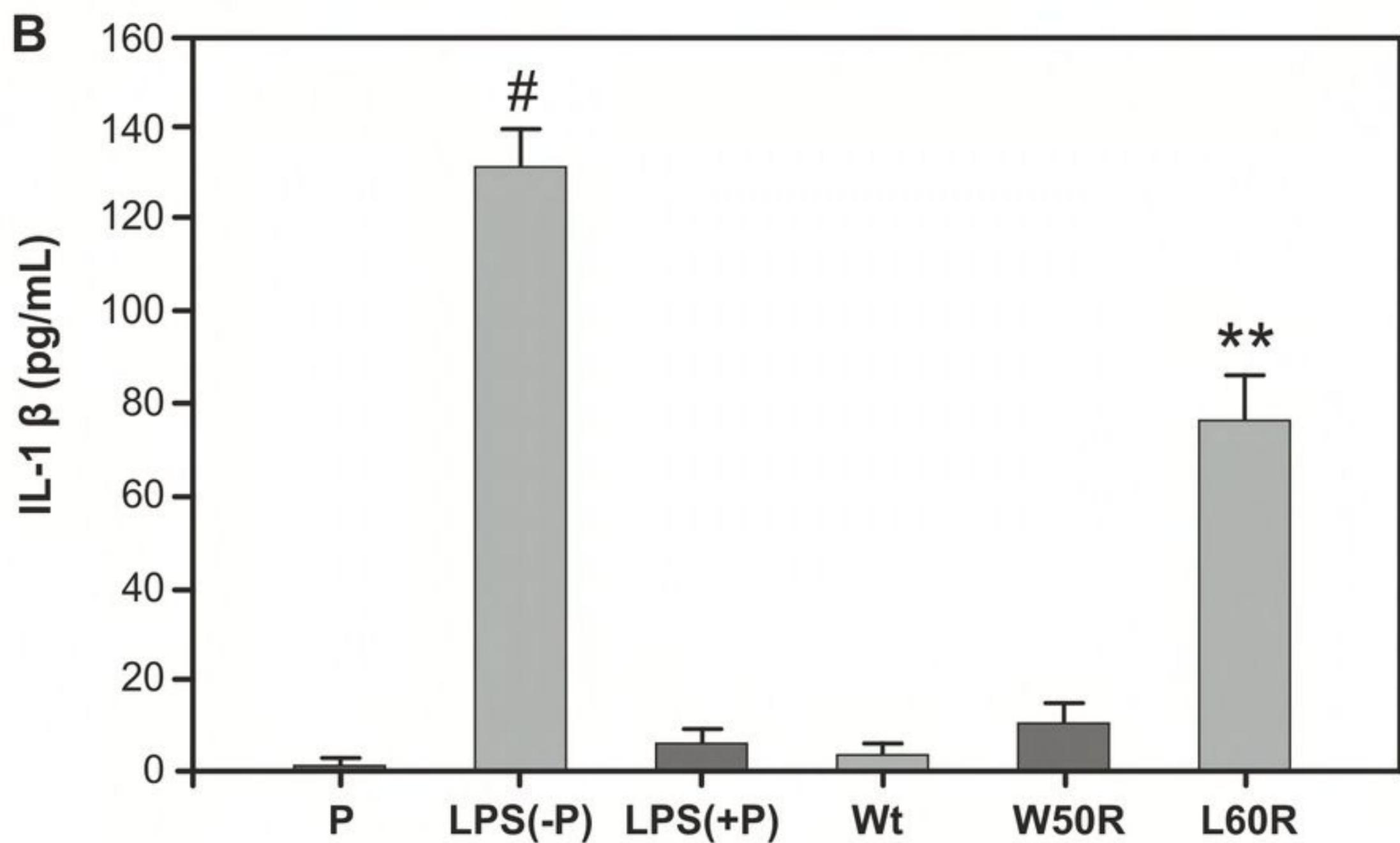
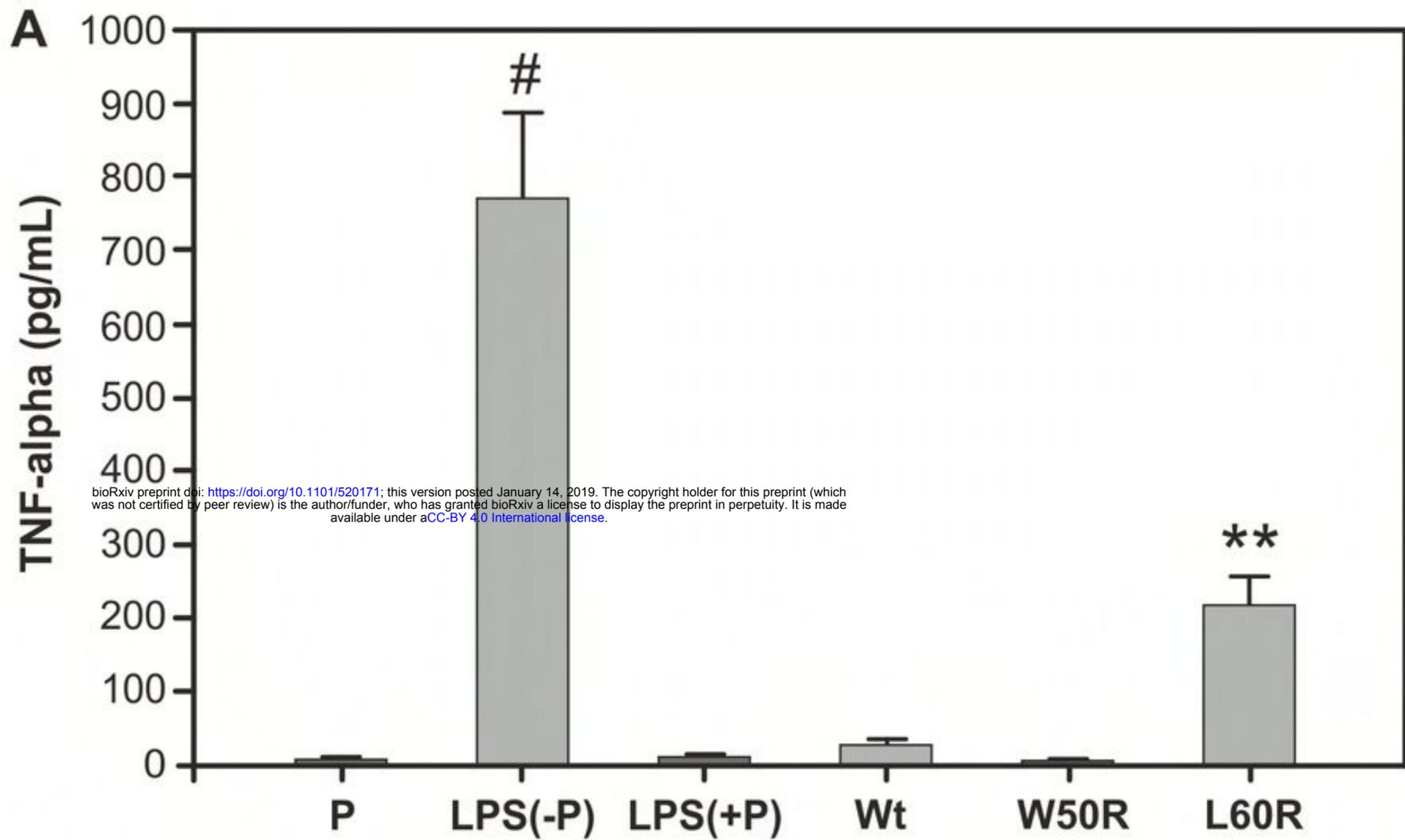


Figure 8

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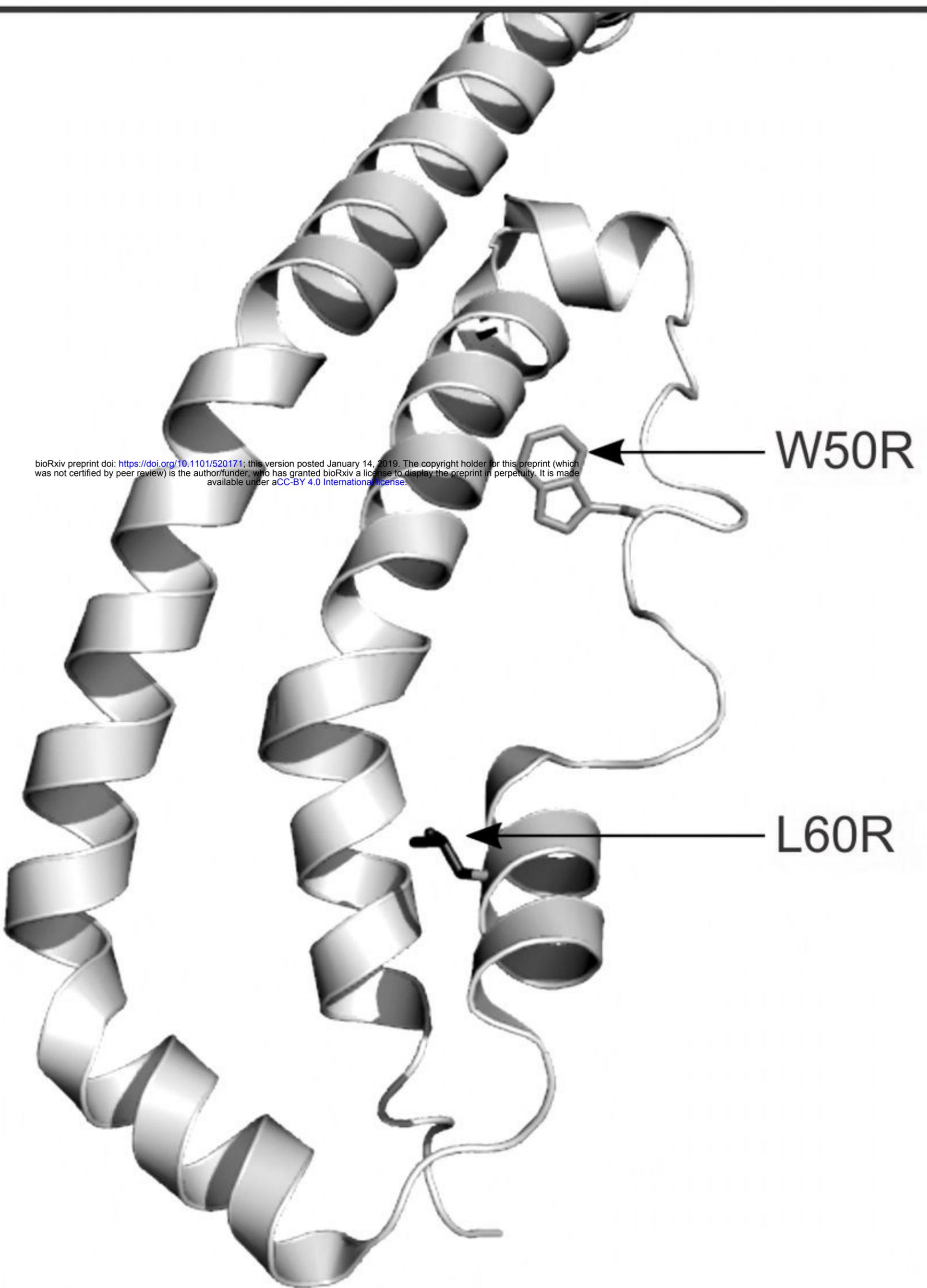


Figure 9

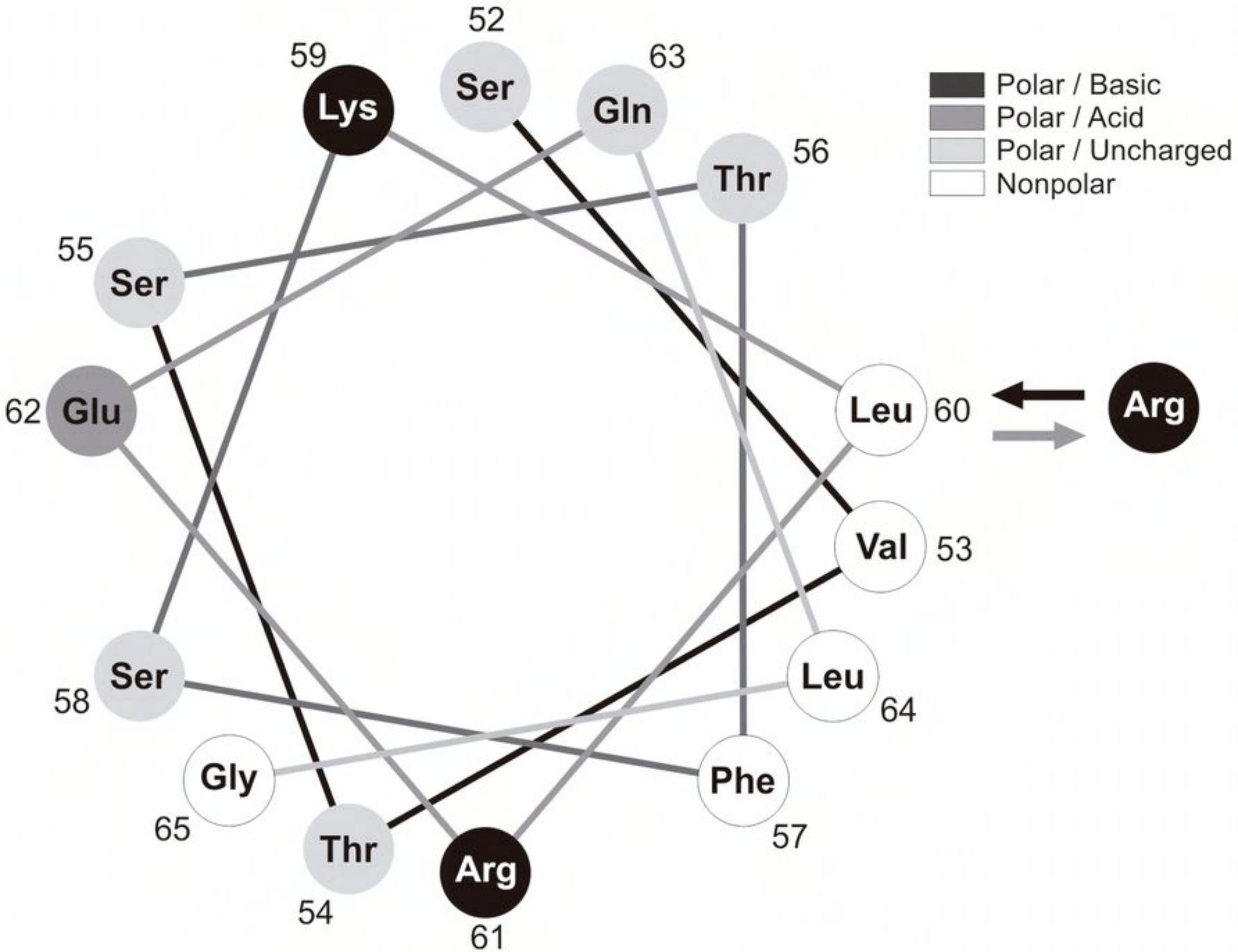


Figure 10