Table 1.

Table 1. Task plan reorganized during reporting period 2 (year 3) as a contingency to ensure good project progress and avoid delay caused by technical difficulties with the full=-lenghtpoA-I recombinant protein production. The most significant deviations from the original plan correspond to the initiation of the work on TTR a year earlier than initially foreseen so that TTR and apoA-I mutants were engineered and produced simultaneously. This was approximately the plan followed throughout the whole project.

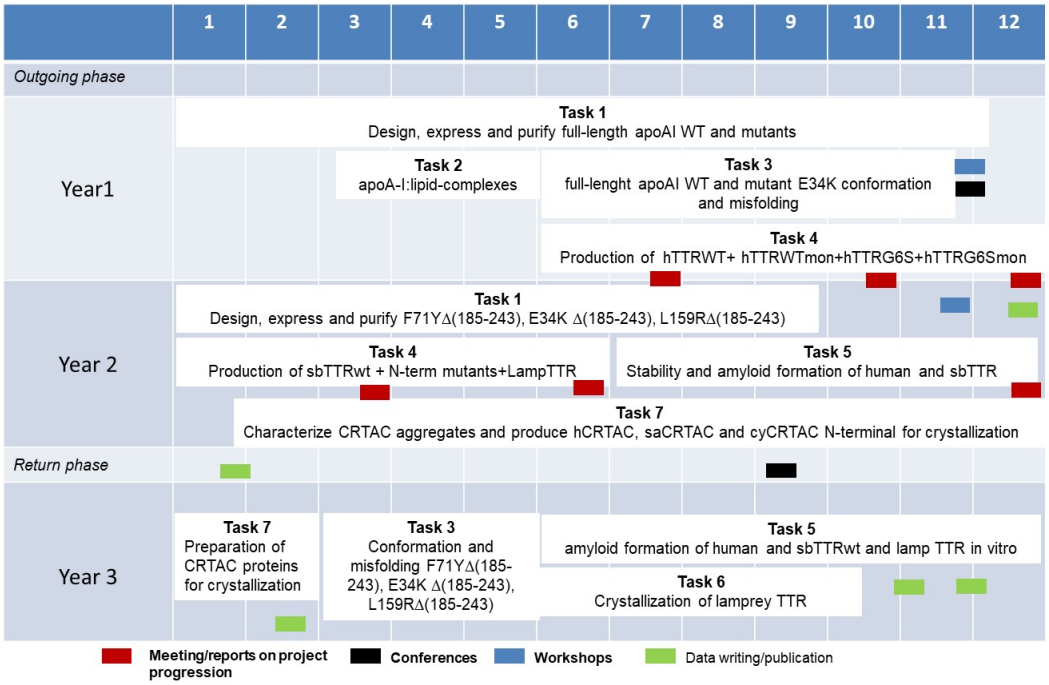

Figure 1

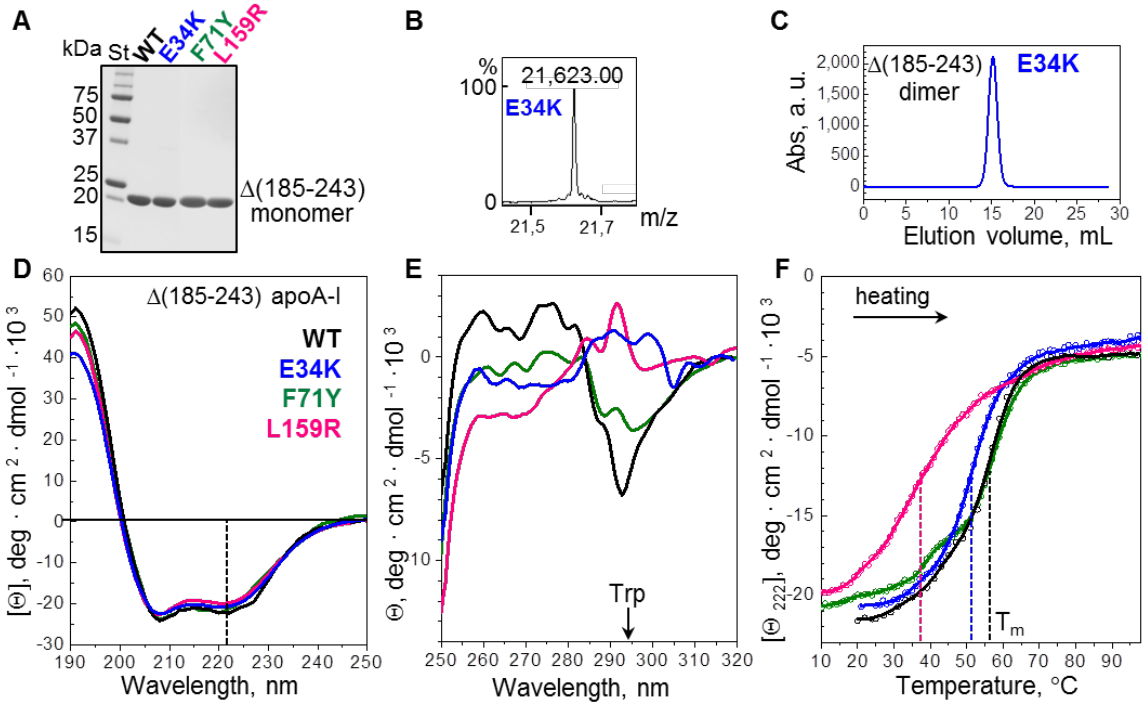

Figure 1. Conformation and stability of apoA-I recombinant mutants and wild type. C-terminally truncated WT (black), E34K (E34K, blue), F71Y (F71Y, green), and L159R (L159R, pink) were produced and their structure and stability analyzed. (A) SDS PAGE (14 % gradient, Denville Blue protein stain) shows that all proteins migrated as a single band with molecular weight ~22 kDa corresponding to Δ(185-243) apoA-I. (B) Intact mass check using electrospray mass spectrometry (shown for E34K) was in excellent agreement with the theoretical molecular weights for the 1-184 fragments of WT (21624,23 Da), E34K (21623,29 Da), F71Y (21640.23) and L159R (21667,22 Da). (C) Size-exclusion chromatography indicates that all truncated proteins migrate as dimers; a representative profile for Δ(185-243) E34K is shown. (D) Far-UV CD spectra indicate a largely α-helical secondary structure in all proteins. Helix content assessed from the CD signal at 222 nm (dashed line) ranged from 56 ± 5% in L159R to 62 ± 5% in WT. Each spectrum represents 3-5 independent measurements. (E) Near-UV CD spectra of point mutants show large differences, particularly at wavelengths 280-305 nm dominated by Trp (peak centered at 295 nm). Each spectrum represents an average of three independent measurements with 5-point adjacent averaging. (F) Melting data recorded by CD at 222 nm, $\Theta_{222}(T)$, monitor α-helical unfolding during heating. Circles show raw data points. Dashed lines indicate melting temperatures, T_m, corresponding to the first derivative maxima, $d\Theta_{222}(T)/dT$.

Figure 2

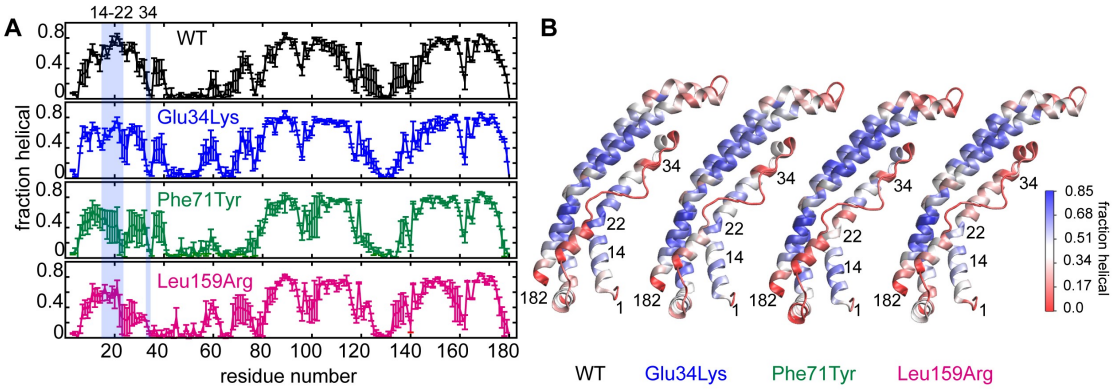

Figure 2. Variations in local helical conformation determined from high-temperature MD simulations. (A) Helical fraction *versus* residue number in apoA-I variants. Standard errors of three replicates are shown by bars. (B) The starting structure for each variant protein is coloured based on the average helicity, from high (blue) to low helical fraction (red). The relative degree of helicity is given by variations in colour intensity as indicated by the coloured bar on the right.

Figure 3.

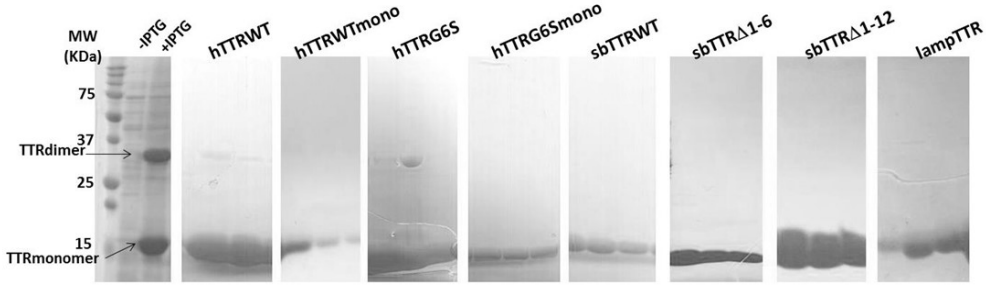

Figure 3. A- SDS-PAGE of hTTRWT expression. Bands are visible for the TTR monomer (12-15KDa) and TTR dimer (24-30 KDa) (shown for hTTR before purification)

Figure 4.

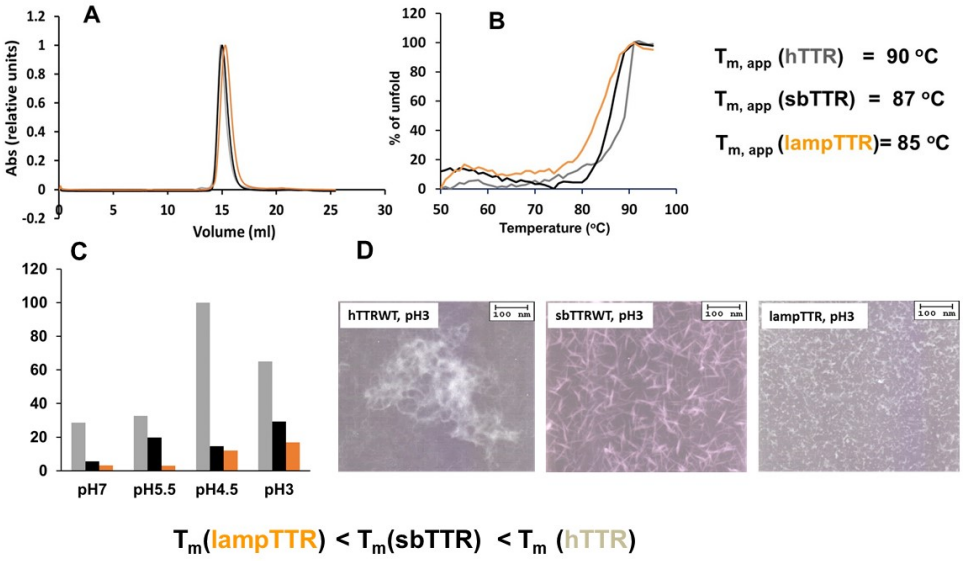

Figure 4. Stability and amyloid propensity of h, sb and lampTTR. A- Size-exclusion chromatography of pure h, sb and lamp TTR showing the presence of a homogeneous tetramer. B- CD scan melt in the presence of Urea 3M. TTRs were heated from 50 to 95oC at 30 oC/h at 220 nm. C-Thioflavin T fluoresce of TTS samples incubated at 37C for 48h.D- Electron microscopy of TTRs samples incubated at 37C for 48h.

Figure 5

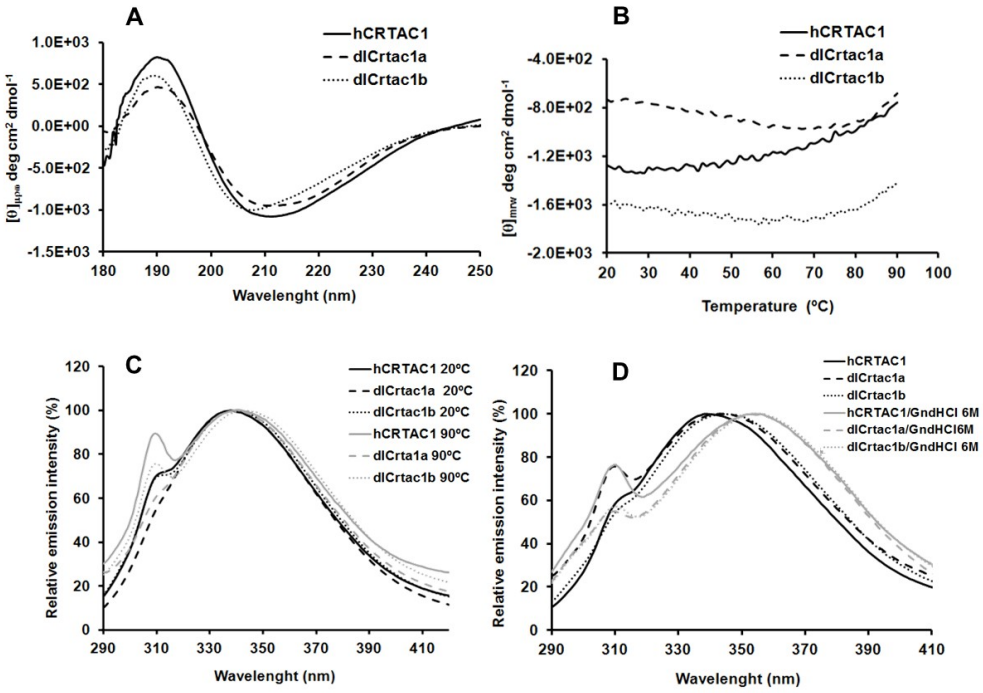

Figure 5. A- Far-UV spectra of recombinant human and piscine CRTAC1's (5uM) in 10mM Tris-HCl pH8 at room temperature (25ºC) scanned from 250 to 180nm. B- Temperature scans of human and piscine CRTAC1's (5uM) ellepticity from 20 to 90ºC at 210 nm in water. C- Intrinsic fluorescence emission spectra of human and piscine CRTAC1's at 20 and 90ºC. D- Intrinsic fluorescence emission spectra of human and piscine CRTAC1's in water with and without pre-incubation in GnHCl 6M.

Figure 6

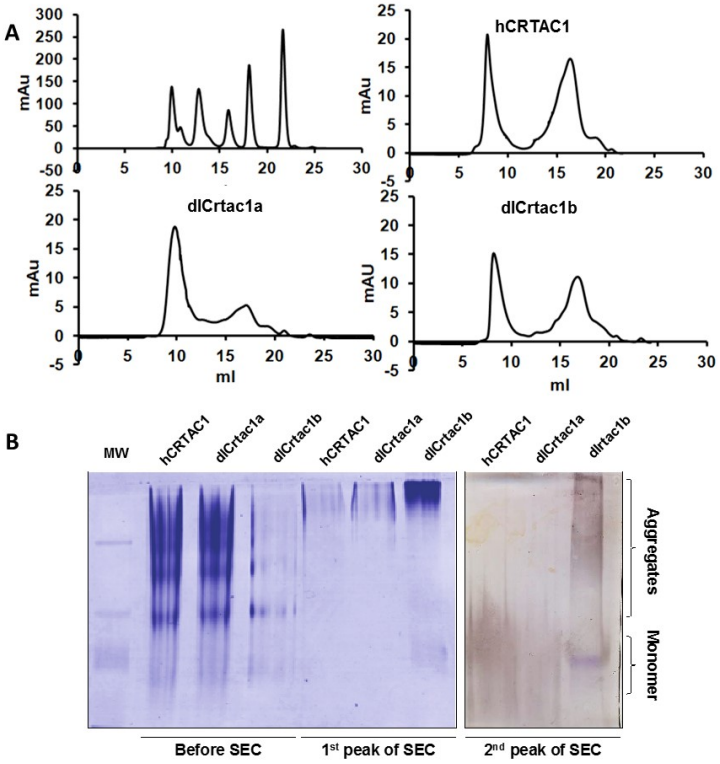

Figure 6. Analysis of human and piscine CRTAC1's aggregation. **A-** Size exclusion chromatographs (SEC) of purified recombinant dlCrtac1a, dlCrtac1b and hCRTAC1. Two main peaks were evident: high molecular weight species that corresponded to protein aggregates (7.7ml for hCRTAC1, 7.9ml for dlCrtac11 and 7.6ml for dlCrtac1b) and a lower molecular weight species that corresponded to monomeric CRTAC1's (14.6 ml for hCRTAC1, 14.7 ml for dlCrtac1a and 15.2 ml for dlCrtac1b). Molecular weight markers used for calibration in the first panel of A were: Thyroglobulin (670 kDa), γ-globulin (158 kDa), Ovalbulmin (44 kDa), Myoglobin (17 kDa), Vitamin B12 (1.35 kDa). **B-** Validation of SEC peak samples for hCRTAC1, dlCrtac1a1 and dlCrtac1b using discontinuous Ornstein-Davis 8% polyacrylamide gel (Native-PAGE). Samples (10 µg) of the 1st and 2nd peaks for each CRTAC1 collected during SEC were mixed with an equal volume of 0.125 M Tris-HCl (pH 6.8), 10% glycerol and 0.01% of bromophenol blue and resolved on a polyacrylamide gel. The gel was stained with Coomassie blue for aggregates (1st peak) and double stained with silver nitrate for monomers (2nd peak).

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Figure 7

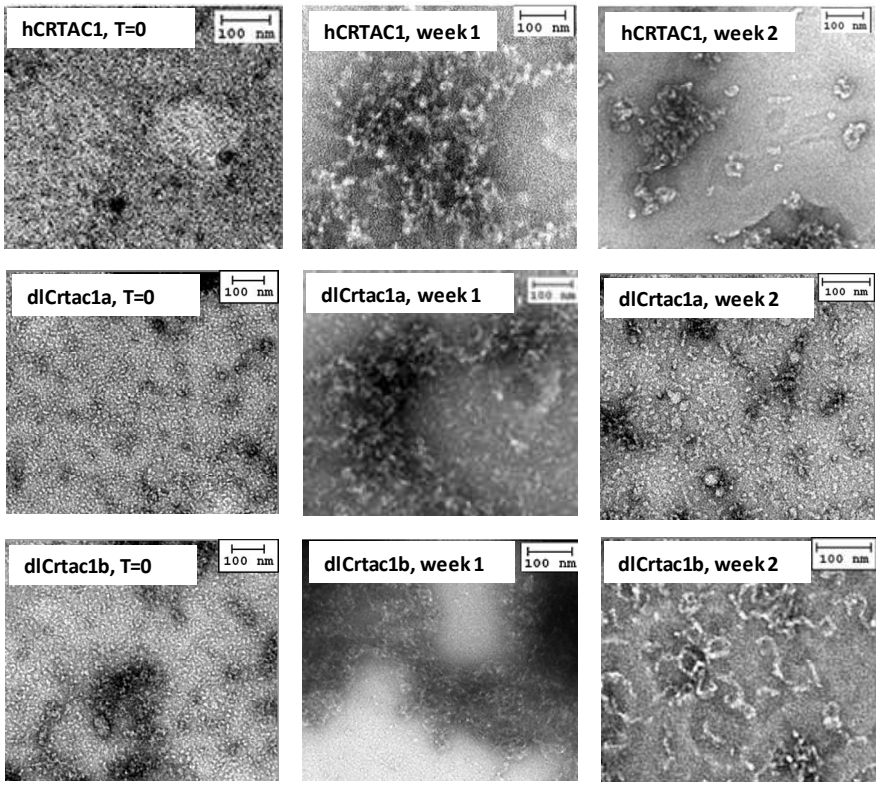

Figure 7. Characterization of CRTAC1's aggregates by A- TEM: transmission electron micrographs of hCRTAC1, dlCrtac1a and dlCrtac1b in 100 mM Tris pH8, 250 mM NaCl before (T=0) and after incubation at 37ºC for one and two weeks.

Figure 8

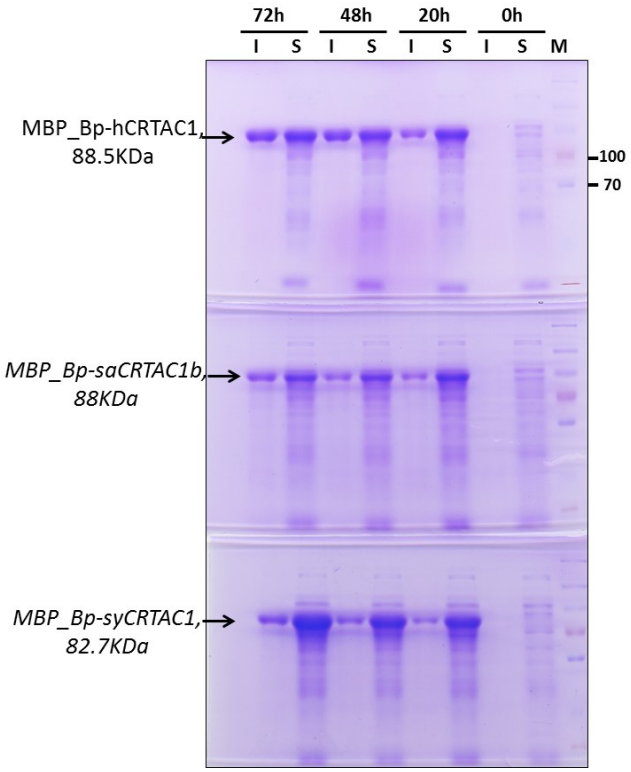

Figure 8. Expression of hCRTAC1, saCRTAC1b and cyCRTAC1 in superior broth at 18C for 72h. Samples were taken at the time points indicated (0h, 20h, 48h and 72h) and analyzed by SDS-PAGE. S-Soluble fraction, I-Insoluble fraction

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