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KIR/KIR-ligand genetic profile in chronic hepatitis B

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Killer **I**mmunoglobulin like **R**eceptors (KIR) are membrane proteins expressed on **N**atural **K**iller (NK) cells and on a small subset of CD8 lymphocytes. They influence the regulation of both cell types through interaction with **H**uman **L**eukocyte **A**ntigen (HLA) class I molecules, activating or inhibiting the innate immune responses.

Several studies have shown that KIR/KIR ligand interactions are involved in the pathogenesis and progression of different diseases as viral infections, autoimmune-disorders, cancer.

The aim of this study is to identify associations between KIR/KIR ligand genetic profile and chronic hepatitis B (CHR).

HBV infection represents a major health problem with 2 billion people infected and 3 hundred and fifty millions people with chronic diseases.

Several studies showed that combinations between KIR and their specific ligands confer susceptibly to or protection against the outcome of the disease.

For this reason, we are conducting a case-control study in Sicilian population,recruiting 24 subjects with positivity to HBcAg, HbE and anticore as cases and 60 "non –exposed" anti-HBc Ag negative subjects as controls.

For the genetic analysis, peripheral blood samples were collected and genomic DNA was extracted from leukocytes. DNA was amplified using PCR-SSP for KIR/KIR ligand genotypization and a size separation was conducted using 2% agarose gel electrophoresis to identify the specific allelic fragments.

Preliminary data showed different frequency of HLA-C2 (p value=0.03), HLA-Bw4^{I} (p value=0.03) and HLA-A (p value<0.00001). Several inhibitory interactions were expressed in CHB. In particular, three of them reach a statistic significance: the association between HLA-A and KIR3DL2 with a p value<0.00001 (strongly significant), the association between KIR2DL2 and HLA-C1 with a p value=0.02 and the association between KIR2DL3 and HLA-C1 with a marginal p value=0.06.

We can conclude that the inhibition of NK cells in subjects with CHB is stronger compared to the general population (HBV negative).

Of course, these data are preliminary results, hence we need to recruit more subjects to characterize in deep the population and to try to identify possible biomarkers to help the physician towards a better diagnosis or a better prediction of the outcome of the diseases.

Vascular risk factors in Mild Cognitive Impairment and Alzheimer's disease: preliminary data from the Zabùt Aging Project

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Background: Dementia is defined as a clinical syndrome characterized by the development of multiple cognitive deficits interfering with daily living, social and professional functioning. Alzheimer's disease (AD) is the most common cause of dementia, accounting for about 60% of cases. Vascular risk factors (VRF) have been described increasing the risk of AD and Mild Cognitive Impairment (MCI), a prodromal construct of dementia and AD. Furthermore, a cluster of vascular disease such as the metabolic syndrome (MetS) has recently been associated with MCI or AD; however, this association has not been confirmed by recent large longitudinal population-based studies.

Objective: to evaluate the correlation between vascular risk factors/diseases and MCI/AD, using population-based data from a Sicilian cohort, the Zabùt Aging Project (ZAP)

Methods. The following dichotomous vascular variables were collected: hypertension, atrial fibrillation, angina, ischemic heart disease, transient ischemic attack (TIA), stroke, diabetes and dyslipidemia. To better evaluate multivariable vascular risk profile, we also evaluated the presence/absence of MetS [1], as well as the 10-year Framingham cardiovascular risk score (FCVS) [2]. In addition, overall comorbidity was assessed by means of the Cumulative Illness Rating Scale (CIRS) [3]. MCI was diagnosed according to the revised Petersen's criteria [4] and AD was diagnosed according to NINCDS-ADRDA [5]. Descriptive data were analyzed by one-way analysis of variance (ANOVA) with Scheffe's post hoc test and Chi-square test with pair comparisons, as appropriate.

Results. Subjects with AD and MCI were older and less educated than control subjects (NCS) (p=<.0001). Furthermore, AD and MCI subjects showed a significantly higher extrapyramidal symptoms burden (p=<.0001) and lower nutritional status (p=<.0001) than NCS. Multisystem comorbidity significantly increased in MCI or AD vs NCS (p=<.0001). Regarding VRF, amnestic MCI (aMCI) showed a higher prevalence of stroke (p=.001) and TIA (p=.03) than NCS. MetS and its specific components didn't vary according to the cognitive diagnosis, whereas FCVS was higher in aMCI than NCS (p=.0008).

Conclusion/Discussion. In general, preliminary data from the ZAP suggested that MCI and AD subjects showed a higher comorbidity compared to NC subjects; however, only aMCI significantly differed comparing to NCS with regards of specific VRF or FCVS. Further analysis of the ZAP cohort, including stratification of subjects by age, sex and APOE genotype can clarify the relationship between VRF and MCI/AD.

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Risk stratification for Splenic Marginal Zone Lymphoma based on IIL Score: correlations between laboratory findings and immunofenotipical expression of B-cell clone and validation on 23 cases.

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Background

Splenic marginal zone lymphoma (SMZL) is a well-defined low-grade lymphoid malignancy recognized as a distinct clinicopathological entity by the WHO 2008 classification of "tumours of haematopoietic and lymphoid tissues". It accounts for about 2% of all lymphoproliferative disorders and represents the vast majority of splenic lymphomas. SMZL is characterized by splenomegaly, moderate lymphocytosis, a typical intrasinusoidal pattern of bone marrow involvement. For SMZL diagnosis, it results necessary the integration of immunophenotypical and biomolecular features and cytogenetic findings. The Intergruppo Italiano Linfomi (ILL) has identified a clinical prognostic score for SMZL, which is able to stratify patients into three different risk categories based on three parameters assessed on diagnosis - anemia, elevated LDH levels and hypoalbuminemia. Despite the indolent nature, one-third of patients experience a rapidly progressive disease, characterized by cytopenias and lymphonode and extranodal involment and may evolve into a high-grade lymphoma. The onset and progression of solid tumors and lymphoid disorders depend on intrinsic genetic aberrations of neoplastic cells and on orchestrated interactions with non-neoplastic bystander (accessory cells such as macrophages or mast cells and immune system cells) which constitute the tumour microenvironment and foster growth and survival of neoplastic elements. A fundamental understanding of these interactions gives insight into the pathogenesis of SMZL and, moreover, may identify novel therapeutic opportunities for targeting oncogenic pathways. Actually in SMZL predictive biological features do not exist and furthermore our research represents a principale objective.

Material and Methods

A series of 23 cases of SMZL according to WHO criteria and in asyntomatic phase, were retrieved from the multicentric study on going IELG-36 BRISMA (Bendamustine and RItuximab for the treatment of Splenic Marginal Zone Lymphoma).

To enlist the patients to this therapeutic protocol, we reviewed the laboratory data, the the peripheral smear and also evaluated the hystological bone marrow features and the immunohistochemical profile to confirm the diagnosis of lymphoma marginal.

Once applied to the score, all patients were stratified in high, intermediate and low risk.

Results

We wanted to test whether features inherent with the peripheral blood analysis were associated with immunophenotipycal clonal expression and if any correlations exist between these variable. To this end we quantified the density of the CD5 on lymphomatous cells, the expression of the heavy chains (IgM+ and IgD+). Furthermore, on multivariate analysis, we detected a correlation between the expression of these markers and IIL score parameters. Actually, among the variables tested, we showed a correlation between low Hemoglobin concentration and CD5 and IgM clonal expression. While no significant associations were detected between hypoalbuminemia and LDH levels. As regarding the risk category, the analysis highlighted the correlation between CD5 and IgM expression and the intermediate/high risk categories. On the base of these results, we can speculate that CD5 and/or IgM expression may be considered as unfavorable prognostic markers to progression disease. However these data may not be considered statistically significant for the number of patients recruited to date. As already known in the literature, B-cell neoplastic clone displays a relevant tropism for the vascular bone marrow niche, we found that, although it is well integrated in the vascular sinusoidal compartment, in few cases it forms pseudo-nodular aggregates, constituted by follicular dendritic cells (FDC), expressing both CD23 and ICAM-1 and generally present within non-Germinal Center neoplastic infiltrates. So we can assessed that the preexisting stroma may be remodeling by the clone and in particular this feature is representative of cases of SMZL in progression toward high-grade lymphoma.

Corso di Dottorato in Medicina Molecolare e Biotecnologie (XXIX Ciclo)-

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A software for patient specific plan verification and Monte Carlo simulations in radiotherapy with proton beams.

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Aims of the project: My research project is essentially based on the implementation of new methodologies related to the commissioning of MedAustron particle therapy facility as regarding both proton and carbon ion beams. The most important part of the facility commissioning is the dosimetric characterization of the beam in all possible clinical conditions and the validation of the software Treatment Planning System TPS (RayStation TPS developed by the RaySearch company) able to compute the dose into the patient geometry.

Methods: The Treatment Planning System (TPS) scripting capability (Python language) has been exploited to develop a new software for the patient specific plan verification. Moreover in order to create a beam model into the TPS, depth dose profiles (Bragg peaks) at different energies need to be measured with plane-parallel ionization chambers. A preliminary investigation of the response of those ionization chambers has been performed by GATE simulation toolkit based on Monte Carlo Geant4.

Results: A software tool based on Python language has been developed and integrated into the TPS with the aim at extracting the dose and the dose gradient at different locations of the PinPoint ionization chambers used for the patient specific plan verification (see Fig. 1).

The response of three plane-parallel ionization chambers (12, 8, 4 cm in diameter) has been evaluated with Monte Carlo simulation at different protons energies. For all three chambers the response depends on the protons energy and the depth in water. Therefore correction factors should be computed in order to compensate the missing charge collection in the finite volume of the plane-parallel ionization chambers.

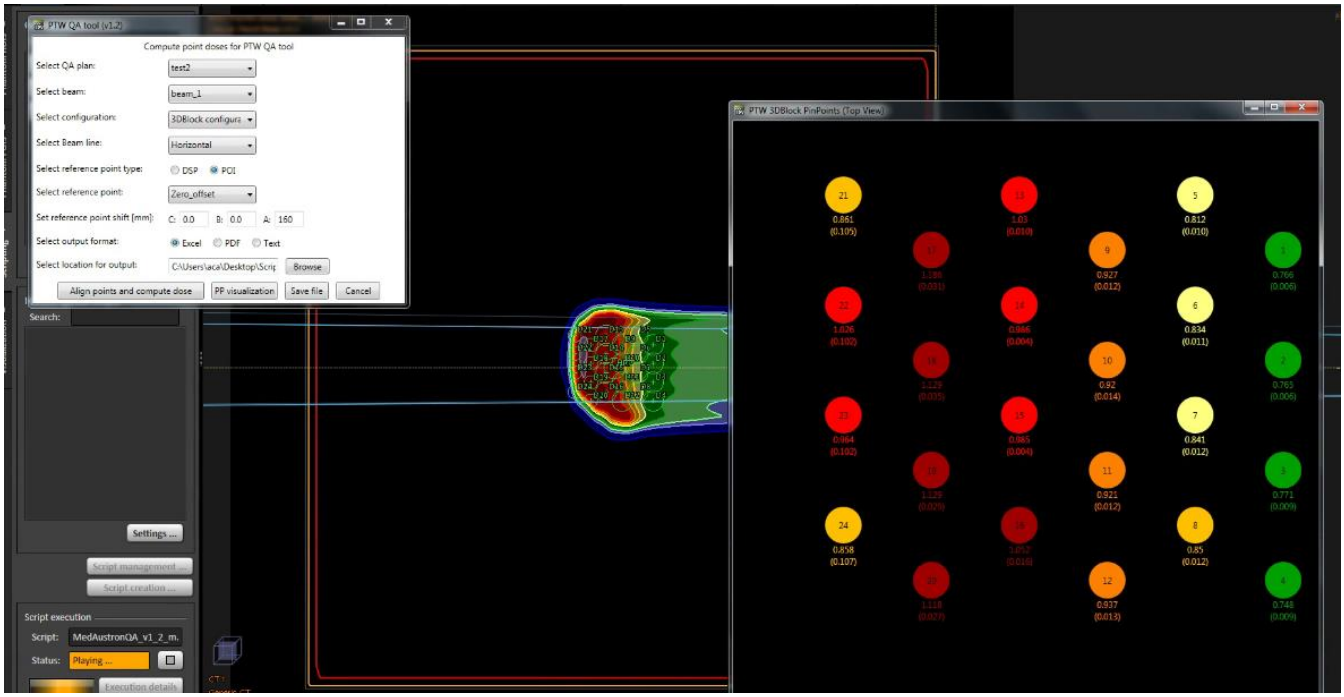

Fig 1: The new software integrated into RayStation TPS pops up a GUI from which the User can visualize the ionization chamber positions, compute and export the corresponding dose values and dose gradients.

Future works: As next steps of my research project:

- Development of a new software for acquisition of the readings of PinPoint ionization chambers, for analysing the measurements and for comparison with the TPS dose distribution.
- Correction factors for the plane-parallel ionization chambers reading will be computed by Monte Carlo simulation.
- A correction for the "quenching effect" of alanine EPR detectors used in the end-to-end test procedures will be computed by means of Monte Carlo simulation.

Genomic and proteomic evaluation of visceral and subcutaneous adipose derived stem cells in a proinflammatory and anti-inflammatory microenvironment

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Adult stem cells can be identified in haematopoietic, neural, gastrointestinal, epidermal and adipose tissues. Adipose derived stem cells (ASCs) are plastic-adherent cells with multilineage capacity, isolated from the stromal vascular fraction (SVF) of adipose tissue. These cells exibit the potential of unlimited proliferation as well as of differentiation along the mesenchymal lineage to produce adipocytes, osteoblasts and chondrocytes.
According to some reports, ASCs might differentiate into pancreatic beta-cells, myocardiocytes and endothelial cells. In addition, ASCs might be used for several clinical applications such as prevention of metabolic diseases (obesity and type 2 diabetes) through the modulation of adipogenesis and lipogenesis, muscle, bone and tissue regeneration, and transplantation.
Aim of the study is the isolation, the phenotypical and functional characterization and the differentiation of human ASCs in a proinflammatory and anti-inflammatory microenvironment and in conditions of glucotoxicity and lipotoxicity.
Subcutaneous and visceral adipose tissue were obtained from 10 normal weight and obese patients (BMI >30 kg/m2) undergoing elective open-abdominal or laparoscopic surgery. Anthropometric (weight, height, BMI, waist circumference, blood pressure and pulse rate) and metabolic parameters (glycaemia, total and HDL cholesterol, triglycerides, blood count) were evaluated. Fat tissue fragments were minced and digested by collagenase, type I. Material was filtered and adipocytes and free oil were separated from stromal vascular components by centrifugation.
The floating fraction was placed in culture flasks filled with DMEM/Ham's F12 1:1 supplemented with 20% fetal bovine serum, and cells were incubated at 37uC in 5% CO2. The primary ASCs grown at the top and bottom of flask, were cultured for 7 days until the confluence (defined as passage 0), and were then split into 60- mm plates. The stromal vascular pellet was resuspended in erythrocyte lysis buffer. The cell suspension was centrifuged and then resuspended in a culture medium consisting of DMEM/Ham's F12, 10% FCS and antibiotics. After a 16-h incubation for cell attachment, cells were cultured in ASCs growth medium. mRNA from visceral and subcutaneous adipose tissues biopsies derived from obese and normal weight patients was isolated by using a RNeasy kit (Qiagen, Hamburg, Germany). ASCs isolated from the subcutaneous and visceral fat depots grew as a characteristic cell monolayer in culture dishes.
Analysis by qRT-PCR for hematopoietic, endothelial and stem cell-associated markers was carried out. The expression of the key ASC markers CD105, CD90, Sox2, Nanog, CD73, ABCG2, Oct4 , c-kit from both subcutaneous and visceral fat depots was observed. Next step are the evaluation of protein expression by western blot, cytofluorimetry and immunofluorescence with expected expression of stem cells markers.

MOLECULAR BASIS OF PRIMARY HYPOBETALIPOPROTEINEIMIAS

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ABSTRACT

Background. Familial hypobetalipoproteinemia is a genetic disorder of lipid metabolism with an autosomal co-dominant inheritance, characterized by plasma levels of total cholesterol, low-density lipoprotein-cholesterol (LDL-C), and apolipoprotein B < 5^{th} percentile of a reference population. FHBL subjects usually show a mild clinical phenotype but they often have hepatic steatosis (fatty liver) and, less frequently, intestinal fat malabsorption. FHBL is genetically heterogeneous; it may be linked to defects in the *APOB* gene or in other genes involved in the assembly, secretion or catabolism of lipoproteins containing apoB. The recent application of NGS has revolutionized the traditional approach of molecular diagnosis with the possibility to parallelize the sequencing process, producing thousands or millions of sequences at the same time. Here it is described the set up of a NGS panel to analyze known candidate genes responsible of FHBL applying the *Ion Torrent* technology.

Methods. The platform *Ion Torrent PGM* (Life Technologies) rely on chemical standard sequencing coupled to a novel detection method based on semiconductor technology designed to detect hydrogen ions released during the DNA polymerization. The design of oligonucleotides panel for amplification of target genes was made using an *in silico* tool, the *Ion AmpliSeq* designer. The workflow based on *Ion AmpliSeq* was used following manufacturer instruction (Life Technologies). Bioinformatics analysis was conducted on the *Ion Reporter* platform, a hardware and software solution, which allows recording data sequencing and annotation of the identified variants.

Results. DNA samples from 6 hypocholesterolemic subjects were analyzed. 2 samples belonging to subjects carrying known mutations were used as positive controls to validate the method. Two libraries were constructed for each DNA sample using suitable primers pool. Variant annotation confirmed the mutations in positive controls. Moreover mutations were identified in three subjects in two candidate genes. In one subject we were not able to identify any mutation. Data were validated and confirmed by Sanger sequencing.

Conclusions and future perspectives. The work showed that the massive sequencing performed using the *Ion Torrent* technology for mutation detection is an accurate and efficient approach of screening mutation responsible of monogenic forms of hypocholesterolemia. The application can be extended to diagnosis of other monogenic diseases and may allow the identification of proband/families in whom other genes could be responsible of the phenotype. These will be candidates to the analysis of whole exome in order to identify novel gene/s involved in these monogenic disorders.

Thermodynamic Characterization on Self-Assembly and Structural Stability of Conditionally Disordered Chaperonins: Hsp60 and GroEL

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Background

Molecular chaperones bind to a large variety of different protein folding intermediates to prevent their non-specific aggregation and facilitate protein folding. Folding chaperones, e.g. Heat shock proteins (Hsp), undergo large conformational rearrangements that modulate client–protein interactions. Some of these conformational changes are associated with Intrinsically Disordered Regions (IDR) [1-3]. Heat shock protein 60kDa is a molecular chaperone (GroEL human homolog) that assists protein folding in mitochondria (mtHsp60). It also plays a role in cytoprotection against cell stressors, displaying for example, antiapoptotic potential [4]. It is synthesized in the cell cytoplasm as a higher molecular weight precursor form (p-mtHsp60) containing an N-terminal targeting sequence, that is cleaved after import into the mitochondrial matrix [5]. It has been established, and demonstrated by various techniques, that Hsp60 can accumulate in the cytosol, in various pathological conditions (i.e., cancer and chronic inflammatory diseases). The cytosolic Hsp60 accumulation mechanism may occur with or without mitochondrial release concomitantly, so that in the cytosol the two types of 60 kDa chaperonin proteins, (mtHsp60 and its precursor naïve form, p-mtHsp60) could coexist [6]. It has been recently observed that in a wide range of concentration, that Hsp60 is able to assemble in both heptamers and tetradecamers [7]. Key questions still unanswered pertain to the differences in structure-function features that might exist between the well-studied prokaryotic GroEL and the largely unexplored eukaryotic Hsp60 proteins. Moreover, studies on human Hsp60 structure and oligomeric state in vitro could help to validate its role in physiological or pathological cases. The research object of this proposal has the aim of starting up the basic knowledge and study of molecular chaperone mechanisms involved in human pathology for the development of a new therapeutic approach. In particular, Alzheimer's Disease (AD), through the development of chaperones able to inhibit β-amyloid (A-β) peptide aggregation.

Results and Conclusion

In order to pursue this goal, we investigated the (dis)assembly and thermal stability of mtHsp60, p-mtHsp60 and GroEL in vitro, by means of Differential Scanning Calorimetry (DSC) and Isothermal Titration Calorimetry (ITC). Complementary Circular Dicroism (CD) measurements were also done to follow the change in the secondary structure due to unfolding.
Results indicate that chaperonins 60kDa exist in a dynamic equilibrium between monomeric, heptameric and tetradeameric form. For both proteins, association involves different endothermic enthalpy and entropy changes. The three Chaperonins have different thermal stability, in fact they unfold with different melting temperatures (Tm), different calorimetric enthalpy changes (ΔHcal) and cooperativity (FWHM). GroEL shows up more stability in respect to Hsp60 and higher cooperativity in the thermal denaturation than Hsp60, in fact GroEL transition occurs within a narrow temperature range. In the case of Hsp60, the transition peak is skewed towards the low temperature side of the transition as expected for a transition coupled to dissociation [8]. Experiments at different protein concentration confirm that the unfolding is coupled to the dissociation of the oligomeric protein.

Moreover, we also investigated a system model chaperone-like, caseins (a very cheap system) , that are known to exert a stabilizing function through direct interaction. We have shown that Caseins have the ability to stabilize proteins, inhibiting the aggregation and the formation of amyloid fibrils, similar to the chaperones of the Heat Shock Proteins (Hsps) [9-10].

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Chemotherapy-related Cardiotoxicity: early identification MR imaging-based

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The project addresses the identification of chemotherapy-related myocardial damage (cardiotoxicity), with cardiac magnetic resonance (CMR).
One of the biggest limits for the use of chemotherapy drugs, both traditional and new-generation, as independent from the oncological problem, is the adversely action (toxic precisely) of those drugs charged to the heart tissue, often irreversible and sometimes lethal. Cardiotoxicity can be understood as the set of adverse cardiac events caused by chemotherapy drugs. Is a growing problem in an aging population resulting in increased incidence of tumors.
Due to cardiotoxicity chemotherapy-related can occur left ventricular dysfunction, heart failure, arrhythmias, coronary arteries disease, etc. So it is really important to have a regular cardiac follow-up for evaluation of left ventricle function.
Few study have shown that CMR may be able to detect cell death due to chemotherapy drugs and to detect subclinical cardiotoxicity, detecting ventricular dysfunction and local fibrosis of the myocardium.
The aim of my project is to improve methods to identify patients at high risk for chemotherapy-related cardiotoxicity which may revolutionize the future of screening and evaluation of these patients.
We aim to analyze at least 60 patients with new tumor diagnosis at a time 0 - 6 - 12 months (after introduction of chemotherapy), for the evaluation of cardiac function and myocardial structural changes using CMR. Today the CMR is considered the gold standard to quantify biventricular function parameters, biventricular global systolic function and myocardial fibrosis. The papers that have been published until today, have focused their attention on any changes in the myocardium using sequences commonly used for the study of biventricular function (the SSFP cine sequences) and the detection of myocardial fibrosis (images captured with T1w IR GRE sequence). We aim to find some earlier signs of myocardial damage using sequences of new generation. Thus we have developed protocol, tested on healthy volunteers, which provides:

- SSFP cine sequences are the sequences normally used to assess biventricular functional parameters,
- T2w-STIR sequence for the identification of areas of myocardial edema,
- T1w IR GRE sequence for the evaluation of Late Gadolinium Enhancement images to detect myocardial fibrosis,
- Tagged MR images for measurements of myocardial motion and characterization of regional myocardial function,
- T1 mapping images for the evaluation of diffuse fibrosis.

Optimization of a biotechnological process for production and purification of two recombinant proteins: Col G and Col H

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Different strategies can be used for increasing production of heterologous recombinant proteins in Escherichia coli. Protein size is often critical for obtaining the best quantity/quality ratio of recombinant protein expression. This study focuses on two recombinant proteins; Class I and class II Collagenases, namely Col G and Col H. Their size is about 140 KDa each. We have developed a method to obtain high levels of cell growth and intracellular expression of each Collagenases in recombinant E. coli BL21(AI). Batch and Fed-batch fermentation procedures have been performed. Results show that Fed-batch technique was most effective in obtaining the highest cell density for each recombinant bacteria; 14/20 gr/l. We also investigated how to optimize recombinant protein expression; best results were obtained when "multiple shot IPTG induction system" was chosen instead of canonical single shot. By applying a purification protocol based on the use of tangential flow filtration and affinity chromatography we were able to obtain the highest quantity of purified protein: about 8.2 gr for Col G and about 7.2 for Col H fermentations. Moreover, by using a stainless steel cooling coil system, we have investigated the effects of low temperature (7°C) during the whole purification process. This system, allowed us to improve the final enzymatic activity of both Collagenases, obtaining 2 fold increase values when measured with Pz Grassmann assay. This study shows that, even when the size of a recombinant protein is limiting, is possible to apply a defined Fed-batch protocol to obtain a very high protein production. Moreover these results ca be used as a scale up starting step for industrial production and purification of these kind of recombinant enzymes.