

ImmunoGuide®

Instructions for Use

Tocilizumab ELISA (mAb-based)

Enzyme immunoassay for the specific and quantitative determination
of free Tocilizumab in serum and plasma

REF: IG-AB108



12X8



2-8°C



AYBAYTECH Biyoteknoloji Ltd. Sti.
Macun Mah. Batı Blv. ATB İş Merkezi No: 1/285,
06374-Yenimahalle, Turkey
Tel : +90 312 397 88 05
Fax : +90 312 397 88 06
e-mail: info@aybaytech.com
www.aybaytech.com

<u>Contents</u>	<u>Page</u>
1. Intended Use	2
2. Summary and Explanation	2
3. Principle of the Test	2
4. Warnings and Precautions	3
5. Storage and Stability of the Kit	3
6. Specimen Collection, Handling and Storage	4
7. Contents of the Kit	4
8. Materials Required but not Supplied	5
9. Procedure Notes	5
10. Pre-Test Setup Instructions	5
10.1. Preparation of Components	5
10.2. Dilution of Standards and Samples	6
11. Test Procedure	6
11.1. General Remarks	6
11.2. Assay Procedure	7
11.3. Quality Control	8
11.4. Calculation of Results	8
12. Assay characteristics	9
12.1. Specificity	9
12.2. Sensitivity	9
12.3. Precision	9
12.4. Recovery	10
13. Automation	10
14. References	10

1. INTENDED USE

Enzyme immunoassay for the specific and quantitative determination of free Tocilizumab in serum and plasma.

2. SUMMARY AND EXPLANATION

The drug Tocilizumab (trade name Actemra[®]) is a humanized monoclonal antibody (mAb) that specifically targets both soluble interleukin-6 receptor (sIL-6R) and membrane bound interleukin-6 receptor (mIL-6R) with high affinity, thereby preventing pro-inflammatory effects of IL-6. The specificity of this test system is achieved by using a monoclonal antibody (clon IG-7C84) for the coating of the microtiter plate and also as a conjugate.

This *ImmunoGuide* Tocilizumab ELISA (mAb-based) is developed for the specific measurement of Tocilizumab in sera, plasma and other biological fluids by the advantage of using a site-directed IG-7C84 mouse monoclonal antibody (mAb) specific for Tocilizumab only. Binding of Tocilizumab to the solid phase, pre-coated with IG-7C84, is inhibited by recombinant human interleukin-6 receptor alpha (rh-IL6R α) in a concentration dependent manner. Therefore, this Tocilizumab ELISA (mAb-Based) measures the free form of Tocilizumab. The choice of specifically measuring the free form allows investigators to analyze the concentration-effect relationship. Due to the use of a mAb as catcher and also as detection reagent this testkit has an unmatched specificity.

3. PRINCIPLE OF THE TEST

This ELISA is based on a Tocilizumab-specific monoclonal antibody (catcher Ab, *ImmunoGuide* clone IG-7C84). Diluted standards and diluted samples are incubated in the microtiter plate coated with IG-7C84 mAb. After incubation, the wells are washed. A horseradish peroxidase (HRP)-conjugated anti-Tocilizumab antibody is added and binds to the Tocilizumab. Following incubation, the wells are washed, and the bound enzymatic activity is detected by addition of chromogen-substrate. The colour developed is proportional to the amount of Tocilizumab in the sample or standard. Results of samples can be determined by using the standard curve. Binding of Tocilizumab to the solid phase, pre-coated with IG-7C84, is inhibited by recombinant human interleukin-6 receptor- α (rh-IL6R α) in a concentration dependent manner. Therefore, the *ImmunoGuide* Tocilizumab ELISA (mAb-Based) measures the free form of Tocilizumab.

4. WARNINGS AND PRECAUTIONS

1. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.
2. In case of severe damage of the kit package, please contact **AybayTech** or your supplier in writing, latest one week after receiving the kit. Do not use damaged components in test runs but keep safe for complaint related issues.
3. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
4. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
5. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
6. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.
7. Avoid contact with Stop solution. It may cause skin irritations and burns.
8. If any component of this kit contains human serum or plasma it is indicated and if so, it has been tested and were found to be negative for HIV I/II, HBsAg and HCV. However, the presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.
9. Some reagents contain preservatives. In case of contact with eyes or skin, flush immediately with water.

5. STORAGE AND STABILITY OF THE KIT

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters. The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2-8°C.

6. SPECIMEN COLLECTION, HANDLING AND STORAGE

Serum, Plasma (EDTA, Heparin)

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	≤-20°C (Aliquots)	Keep away from heat or direct sun light Avoid repeated freeze-thaw cycles
Stability:	3 d	6 mon	

7. CONTENTS OF THE KIT

QUANTITY	COMPONENT
1 x 12 x 8	Microtiter Plate Break apart strips coated with anti-Tocilizumab monoclonal antibody.
5 x 0.5 mL	Tocilizumab Standards A-E, Concentrate (10X) 2000; 600; 200; 60; and 0 ng/mL Used for construction of the standard curve. Contains Tocilizumab, proteins, preservative and stabilizer.
1 x 12 mL	Assay Buffer Blue colored. Ready to use. Contains proteins and preservative.
1 x 60 mL	Dilution Buffer, Concentrate (5X) Contains orange dye, proteins and preservative.
1 x 12 mL	Enzyme Conjugate Red colored. Ready to use. Contains horseradish peroxidase(HRP)-conjugated anti-Tocilizumab monoclonal antibody, Proclin [®] and stabilizers.
1 x 12 mL	TMB Substrate Solution Ready to use. Contains 3,3',5,5'-Tetramethylbenzidine (TMB).
1 x 12 mL	Stop Solution Ready to use. 1 N Hydrochloric acid (HCl).
1 x 50 mL	Wash Buffer, Concentrate (20x) Contains buffer, Tween [®] 20 and Kathon [™] .
2 x 1	Adhesive Seal For sealing microtiter plate during incubation.

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 μL .
2. Bidistilled or deionised water and calibrated glasswares (e.g. flasks or cylinders).
3. Wash bottle, automated or semi-automated microtiter plate washing system
4. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength at 600-650 nm is optional).
5. Absorbent paper towels, standard laboratory glass or plastic vials, and a timer.

9. PROCEDURE NOTES

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pre-treatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared readily at the appropriate time. Allow all reagents and specimens to reach room temperature (20-25 $^{\circ}\text{C}$) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange the caps of vials. Always cap not used vials. Do not reuse wells or reagents.
4. Use a pipetting scheme to verify an appropriate plate layout.
5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
7. Humidity affects the coated wells. Do not open the pouch until it reaches room temperature. Unused wells should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of Components*

Dilute/ dissolve	Component		Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer	up to 200 mL	Distilled Water	1:20	Warm up at 37 $^{\circ}\text{C}$ to dissolve crystals. Mix vigorously.	2-8 $^{\circ}\text{C}$	4 w
10 mL	Dilution Buffer	up to 50 mL	Distilled Water	1:5		2-8 $^{\circ}\text{C}$	4 w

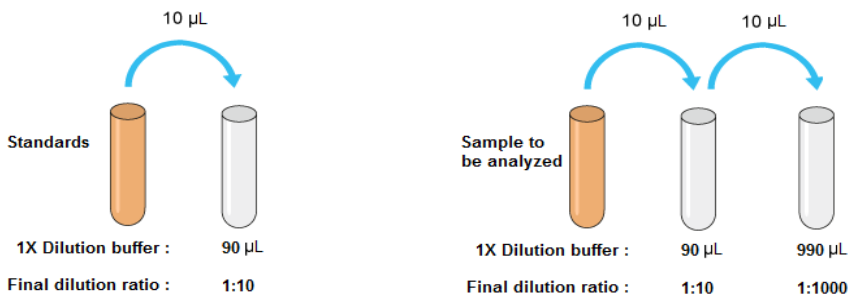
* Prepare Wash and Dilution Buffers before starting the assay procedure.

10.2. Dilution of Standards and Samples

The dilutions depicted below are examples of how to obtain final dilutions for standards and samples. Standards and samples should be properly diluted as homogenous mixture before starting the assay procedure. It is recommended mixing the standards and samples well to homogenous solution at each dilution step. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

1. 10 μL of standard or sample added to 90 μL of 1X dilution buffer, giving the total volume of 100 μL and a dilution of 1:10.
2. 10 μL of 1:10 diluted sample added to 990 μL of 1X dilution buffer, giving the total volume of 1000 μL and a final dilution of 1:1000. This 2nd dilution should not be done with the standards.
3. Samples with a drug concentration above the measuring range should be rated as “>highest standard”. The result should not be extrapolated. The sample in question should be further diluted with 1X Dilution Buffer and then retested.

Standard/Sample Dilution



11. TEST PROCEDURE

11.1. GENERAL REMARKS

11.1.1. Before performing the assay, samples and assay kit should be brought to room temperature (about 30 minutes beforehand) and ensure the homogeneity of the solution.

11.1.2. All Standards should be run with each series of unknown samples.

11.1.3. Standards should be subject to the same manipulations and incubation times as the samples being tested.

11.1.4. All steps of the test should be completed without interruption.

11.1.5. Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination.

11.1.6. The total pipetting time needed for dispensing all samples into the wells should not exceed 5 minutes. If this is difficult to achieve the samples should be pre-dispensed in a separate neutral polypropylene microplate and then transferred into the reaction ELISA plate by a multi channel pipette.

11.2. ASSAY PROCEDURE

1.	Pipette 100 μL of Assay Buffer into each of the wells to be used.
2.	Pipette 75 μL of each 1:10 Diluted Standard, and 1:1000 Diluted Samples into the respective wells of the microtiter plate. Bubble formation during the pipetting of standards and samples must be avoided. <u>Wells</u> A1: Standard A B1: Standard B C1: Standard C D1: Standard D E1: Standard E F1 and so on: Samples (Serum/Plasma)
3.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 60 min at room temperature (RT, 20-25°C).
4.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 350 μL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
5.	Pipette 100 μL of Enzyme Conjugate (HRP-anti Tocilizumab) into each well.
6.	Cover the plate with adhesive seal. Shake plate carefully by tapping several times. Incubate the plate on a bench top for 60 min at room temperature (RT, 20-25°C).
7.	Remove adhesive seal. Aspirate or decant the incubation solution. Wash the plate 3 X 350 μL of Diluted Wash Buffer per well. Remove excess solution by tapping the inverted plate on a paper towel.
8.	Pipette 100 μL of Ready-to-Use TMB Substrate Solution into each well.
9.	Incubate 15 min at RT. Avoid exposure to direct sunlight.
10.	Stop the substrate reaction by adding 100 μL of Stop Solution into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow.
11.	Measure optical density (OD) with a photometer at 450 nm (Reference at OD620 nm is optional) within 15 min after pipetting the Stop Solution.

11. 3. QUALITY CONTROL

The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards/controls must be found within the acceptable ranges as stated above and/or label. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation, the following technical issues should be reviewed: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

11. 4. CALCULATION OF RESULTS

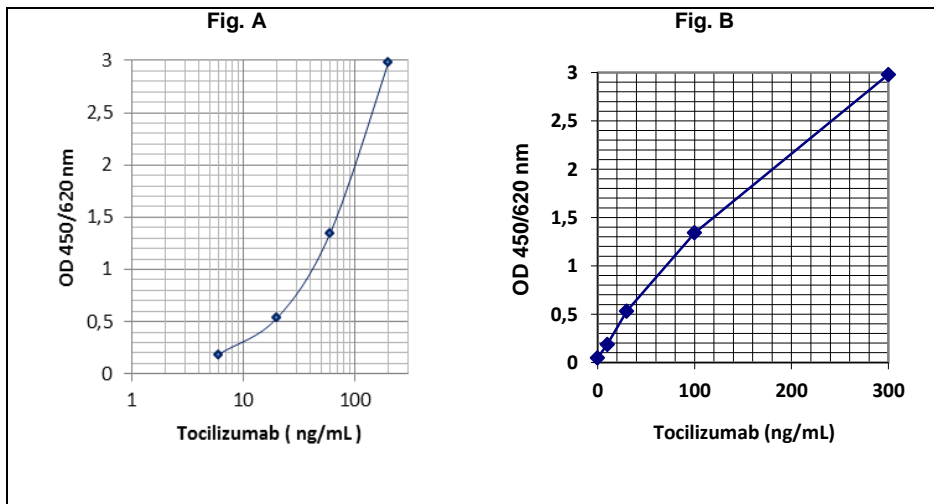
A standard curve should be constructed using the standard concentration (X-axis) versus the OD450 (or OD450/620) values (Y-axis). This can be done manually using graph paper or with a computer program. Concerning the data regression by computer, it is recommended to primarily use the "4 Parameter Logistic (4PL)" or alternatively the "point-to-point calculation". In case of manual plot there are 2 options: Semilog graph (see Fig. A) or linear graph (see Fig. B). Semilog graph paper is available at <http://www.papersnake.com/logarithmic/semilogarithmic/>.

The concentration of the samples can be read from this standard curve as follows. Using the absorbance value for each sample, determine the corresponding concentration of the drug from the standard curve. This value always has to be multiplied by the individual dilution factor (usually 1000). If any diluted sample is reading greater than the highest standard, it should be further diluted appropriately with 1X Dilution Buffer and retested. Also this second dilution has to be used for calculation of the final result. We are recommending that each laboratory determines the best initial dilution for their samples in order to minimize retesting.

Typical Calibration Curve

(All steps were performed at 23°C. Just an example. Do not use it for calculation!)

1:10 Diluted Standard	A	B	C	D	E
Concentration (ng/mL)	200	60	20	6	0
Mean OD _{450/620 nm}	2.979	1.342	0.532	0.187	0.047



12. ASSAY CHARACTERISTICS

12.1. SPECIFICITY

There is no cross reaction with any other proteins present in native human serum. A screening test was performed with 32 different native human sera. All produced OD_{450/620nm} values less than the mean OD of standard D (6 ng/mL). No cross reaction was observed with sera spiked with the other therapeutic antibodies including Adalimumab, Golimumab, Infliximab, Etanercept, Rituximab, Bevacizumab, Trastuzumab and Omalizumab at concentrations up to 1 mg/mL. All produced OD_{450/620nm} values less than the mean OD of standard D.

12.2. SENSITIVITY

The lowest detectable level that can be clearly distinguished from the zero standard is 2 ng/mL (zero standard +2SD read from the curve) under the above-described conditions. Analytical sensitivity is 2 ng/mL, and corresponding to the detection limit (limit of quantification) of 2 µg/mL for undiluted clinical samples because the serum or plasma samples are instructed to be diluted at 1:1000 before starting the assay.

12.3. PRECISION OF THE KIT

Intra-assay CV: <10%.

Inter-assay CV: <10%.

12.4. RECOVERY

Recovery rate was found to be >95% with native human serum and plasma samples when spiked with exogenous Tocilizumab.

13. AUTOMATION

The *ImmunoGuide* Tocilizumab ELISA (mAb-based) is suitable also for being used by an automated ELISA processor.

14. REFERENCES

1. Inciarte-Mundo J, Ruiz-Esquide V, Hernández MV, Cañete JD, Cabrera-Villalba SR, Ramirez J, Yagüe J, Sanmarti R. Calprotectin more accurately discriminates the disease status of rheumatoid arthritis patients receiving tocilizumab than acute phase reactants. *Rheumatology (Oxford)*. 2015 Aug 4. pii: kev251. [Epub ahead of print]
2. Kennedy GA, Varelias A, Vuckovic S, Le Texier L, Gartlan KH, Zhang P, Thomas G, Anderson L, Boyle G, Cloonan N, Leach J, Sturgeon E, Avery J, Olver SD, Lor M, Misra AK, Hutchins C, Morton AJ, Durrant ST, Subramoniapillai E, Butler JP, Curley CI, MacDonald KP, Tey SK, Hill GR. Addition of interleukin-6 inhibition with tocilizumab to standard graft-versus-host disease prophylaxis after allogeneic stem-cell transplantation: a phase 1/2 trial. *Lancet Oncol*. 2014;15(13):1451-9
3. Besada E. Potential patient benefit of a subcutaneous formulation of tocilizumab for the treatment of rheumatoid arthritis: a critical review. *Patient Prefer Adherence*. 2014;8:1051-9.
4. Ohta S, Tsuru T, Terao K, Mogi S, Suzuki M, Shono E, Ishida Y, Tarumi E, Imai M. Mechanism-based approach using a biomarker response to evaluate tocilizumab subcutaneous injection in patients with rheumatoid arthritis with an inadequate response to synthetic DMARDs (MATSURI study). *J Clin Pharmacol*. 2014;54(1):109-19.
5. Lee SJ, Park W, Park SH, Shim SC, Baek HJ, Yoo DH, Kim HA, Lee SK, Lee YJ, Park YE, Cha HS, Park JK, Lee EY, Lee EB, Song YW. Low baseline interleukin-17A levels are associated with better treatment response at 12 weeks to tocilizumab therapy in rheumatoid arthritis patients. *J Immunol Res*. 2015;2015:487230. doi: 10.1155/2015/487230. Epub 2015 Apr 2
6. Ogata A, Tanimura K, Sugimoto T, Inoue H, Urata Y, Matsubara T, Kondo M, Ueki Y, Iwahashi M, Tohma S, Ohta S, Saeki Y, Tanaka T; Musashi Study Investigators. Phase III study of the efficacy and safety of subcutaneous versus intravenous tocilizumab monotherapy in patients with rheumatoid arthritis. *Arthritis Care Res (Hoboken)*. 2014;66(3):344-54.
7. Mori S, Ueki Y. Primary lack of efficacy of infliximab therapy for rheumatoid arthritis: pharmacokinetic characterization and assessment of switching to tocilizumab. *Mod Rheumatol*. 2011;21(6):628-36.
8. Zhang X, Peck R. Clinical pharmacology of tocilizumab for the treatment of patients with rheumatoid arthritis. *Expert Rev Clin Pharmacol*. 2011 Sep;4(5):539-58. doi: 10.1586/ecp.11.33.
9. Burmester GR, Rigby WF, van Vollenhoven RF, Kay J, Rubbert-Roth A, Kelman A, Dimonaco S, Mitchell N. Tocilizumab in early progressive rheumatoid arthritis: FUNCTION, a randomised controlled trial. *Ann Rheum Dis*. 2015 Oct 28. pii: annrheumdis-2015-207628. doi: 10.1136/annrheumdis-2015-207628. [Epub ahead of print]
10. Gabay C, Riek M, Hetland ML, Hauge EM, Pavelka K, Tomšič M, Canhao H, Chatzdzionysiou K, Lukina G, Nordström DC, Lie E, Ancuta I, Hernández MV, van Riel PL, van Vollenhoven R, Kvien TK. Effectiveness of tocilizumab with and without synthetic disease-modifying antirheumatic drugs in rheumatoid arthritis: results from a European

- collaborative study. *Ann Rheum Dis*. 2015 Sep 15. pii: annrheumdis-2015-207760. doi: 10.1136/annrheumdis-2015-207760
11. Espinoza F, Jorgensen C, Pers YM. Efficacy of Tocilizumab in the treatment of Eosinophilic fasciitis: Report of one case. *Joint Bone Spine*. 2015 Jul 7. pii: S1297-319X(15)00101-3. doi: 10.1016/j.jbspin.2015.02.008. [Epub ahead of print]
12. Roddy JV, Haverkos BM, McBride A, Leininger KM, Jaglowski S, Penza S, Klisovic R, Blum W, Vasu S, Hofmeister CC, Benson DM, Andritsos LA, Devine SM, Efebera YA. Tocilizumab for steroid refractory acute graft-versus-host disease. *Leuk Lymphoma*. 2015 Jul 3:1-5. [Epub ahead of print] 9.
13. Kim NH, Kim SK, Kim DS, Zhang D, Park JA, Yi H, Kim JS, Shin HC. Anti-proliferative action of IL-6R-targeted antibody tocilizumab for non-small cell lung cancer cells. *Oncol Lett*. 2015;9(5):2283-2288.
14. Hammoudeh M, Al Awadhi A, Hasan EH, Akhlaghi M, Ahmadzadeh A, Sadeghi Abdollahi B Safety, Tolerability, and Efficacy of Tocilizumab in Rheumatoid Arthritis: An Open-Label Phase 4 Study in Patients from the Middle East. *Int J Rheumatol*. 2015;2015:975028. doi: 10.1155/2015/975028.
15. Yang L, Xing R, Li C, Liu Y, Sun L, Liu X, Wang Y. Active immunization with Tocilizumab mimotopes induces specific immune responses. *BMC Biotechnol*. 2015 Jun 2;15:46. doi: 10.1186/s12896-015-0161-9.
16. Burmester GR, Rubbert-Roth A, Cantagrel A, Hall S, Leszczynski P, Feldman D, Rangaraj MJ, Roane G, Ludvico C, Bao M, Rowell L, Davies C, Mysler EF. Efficacy and safety of subcutaneous tocilizumab versus intravenous tocilizumab in combination with traditional DMARDs in patients with RA at week 97 (SUMMACTA). *Ann Rheum Dis*. 2015 Jun 8. pii: annrheumdis-2015-207281. doi: 10.1136/annrheumdis-2015-207281.
17. Romão VC, Santos MJ, Polido-Pereira J, Duarte C, Nero P, Miguel C, Costa JA, Bernardes M, Pimentel-Santos FM, Barcelos F, Costa L, Melo Gomes JA, Pereira da Silva JA, Cunha Branco J, Canas da Silva J, Pereira da Silva JA, Fonseca JE, Canhão H. Comparative Effectiveness of Tocilizumab and TNF Inhibitors in Rheumatoid Arthritis Patients: Data from the Rheumatic Diseases Portuguese Register, Reuma.pt. *Biomed Res Int*. 2015;2015:279890. doi: 10.1155/2015/279890
18. Wang G, Mu R, Xu H. Management of rheumatoid arthritis in People's Republic of China - focus on tocilizumab and patient considerations. *Int J Gen Med*. 2015;8:187-94.
19. Bensaoud N, Rostom S, Bahiri R, Hajjaj-Hassouni N. Efficacy of tocilizumab on MRI-determined bone oedema in rheumatoid arthritis. *Clin Rheumatol*. 2015;34(6):1031-7.
20. Narváez J, Díaz-Torné C, Magallares B, Hernández MV, Reina D, Corominas H, Sanmartí R, de la Serna AR, Llobet JM, Nolla JM. Comparative effectiveness of tocilizumab with either methotrexate or leflunomide in the treatment of rheumatoid arthritis. *PLoS One*. 2015 Apr 1;10(4):e0123392. doi: 10.1371/journal.pone.0123392. eCollection 2015.